DEVELOPMENTAL VARIATIONS IN THE PERIPHERAL ERYTHROCYTIC SYSTEM OF THE RAINBOW TROUT, *Salmo gairdneri*

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ABSTRACT

The peripheral circulating erythrocytic system of the rainbow trout, Salmo gairdneri, was examined in vitro in relation to differences in the morphology and multiple hemoglobin system organization of adult and juvenile red cells.

Cells were separated by velocity sedimentation under unit gravity, a procedure requiring red cell exposure to an incubation medium for periods of at least three hours. Therefore, this must provide an environment in which red cells remain in a condition approximating normalcy. Previous studies having demonstrated commonly employed media to be ineffective in this regard, a medium was developed through modification of Cortland saline. One of the principal additions to this medium, norepinephrine, altered cell regulation of intracellular calcium, magnesium and chloride concentrations. Catecholamine involvement was also suggested in the synthesis of hemoglobin.

The procedure was found to separate cells primarily by density and, to a lesser extent, by shape. Characterization of red cells revealed two subpopulations to exist. The first comprised the bulk of the cell population, and were of greater length, width, volume and major:minor axis ratio than the smaller population; these were adult cells. The latter, juvenile cells were of smaller overall size and were more spherical in shape. Juvenile cells also possessed fewer electrophoretically-distinguishable isomorphs than did adults with only eight of eleven hemoglobin components typically found. With maturation,

hemoglobin complement with the development of three more bands. The total complement of the adult cell contained 7 cathodal bands and four anodal hemoglobin isomorphs. Bands acquired with maturation comprised the smallest percentage of the cells hemoglobin, each averaging less than one-percent of the total. Whether these additional bands are derived through degradation and reaggregation of existing components or are the product of de novo synthesis is not yet known.

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INTRODUCTION

Freshwater fishes are regularly confronted with changing environmental parameters which influence oxygen uptake at respiratory surfaces and/or oxygen demand by working muscle and other tissues. A number of mechanisms have evolved at the hematological level to meet these changing needs.

Variations in the ionic environment provided by the red cell can influence hemoglobin configuration, either directly through binding with the respiratory pigment (e.g., H^+, Cl^-, ATP, GTP, possibly Na^+, K^+) or by complexation with molecular compounds that modify hemoglobin structure (e.g. Mg^{+2} and Ca^{+2} binding to organophosphate modulators) (Perutz, 1978). Oxygen affinity is altered as a consequence and, dependent upon the magnitude and nature of configurational change, oxygen binding curves exhibit a lateral shift (Perutz, 1978).

These effects are potentiated in a species such as the rainbow trout, Salmo gairdneri, which form functionally-heterogeneous hemoglobin systems. Each hemoglobin is electrophoretically distinct. Some isoforms are more effective than others under particular conditions in oxygen uptake at the gills and/or release to oxygen-depleted tissues (Weber et al, 1976; Houston, 1980). Consequently, adaptive changes in the specific isoform abundancies can lead to a predominance of those forms which are better suited to oxygen transport under existing conditions while reducing the relative importance of those operating suboptimally (Tun, 1985).

A third form of hematological response involves increasing

the hemoglobin content of whole blood. This can be accomplished either through the addition of more blood cells to circulating blood from storage/formation sites such as spleen and head kidney or by induced maturation of cells already present in circulation. The latter leads to an increase in mean cell hemoglobin content through de novo protein synthesis without an increase in cell number and overall blood viscocity (Houston, 1980).

Whether or not these changes take place in all circulating cells or only in a subpopulation of the circulating erythrocytic spectrum is not yet known. Unlike the situation in the mammalian peripheral circulatory system, teleostean red cells are not released to circulation from hematopoietic sites in fully adult form. Instead they mature while in circulation, this being possible as the cells are nucleated and have a lifespan exceeding 240 days (Iuchi, 1973b).

It would seem conceivable that the hemoglobin complement of immature red cells might not be fully determined in terms of either the number of hemoglobin isomorphs or the relative importance of each. Indeed, Iuchi and Yamagami (1969) have reported that the blood cells of larval Salmo gairdneri have fewer and different hemoglobins than the red cells of adults. They hypothesized that as the fish matures, larval red cells are replaced by a transitional cell type which is in turn replaced by mature erythrocytes. A similar situation may exist in the red cell population of adult trout. Circulating juvenile cells may form appropriate hemoglobins in response to alterations in the environment, modifying overall isoform abundancies. Alterna-

tively, erythropoiesis and hemoglobin synthesis and accumulation may be stimulated. Passive reaggregation of existing globin subunits in mature erythrocytes to form new isoforms is also possible. However, it is unlikely that the latter cells responsively synthesize new globins; most of the organelle complement is lost during maturation (Brunner et al, 1977).

While whole animal studies are useful in determination of hematological response to respiratory demands, they often do not permit confident interpretation of the mechanisms underlying these changes. It becomes a nearly impossible task to assess all of the factors influencing the status of circulating erythrocytes and an even more difficult problem to evaluate their relative effects. Under such circumstances in vitro approaches are generally employed as the composition of the incubation medium and response to manipulation can be followed in a more orderly fashion.

Application of in vitro approaches to the study of in vivo cellular processes involves a crucial assumption: that erythrocytes in culture approximate a functionally normal condition. Recent studies (Greaney and Powers, 1978; Bourne and Cossins, 1982, 1984) have shown that this is not always the case. Significant changes in ATP content have been observed and ionic composition changes markedly during incubation. A more detailed study (Houston et al, 1985) of two commonly employed commercial media, Eagle's minimum essential medium and Cortland saline, demonstrated significant variations in nucleotide triphosphate (NTP) levels, water content and both anionic and cationic

concentration during incubation. These changes would be expected to alter hemoglobin configuration and to have profound effects on normal erythrocyte function. In short, the provision of an incubation medium in which red cells will remain in a relatively normal state is essential prior to more detailed studies on mechanisms of cellular adaptation.

Following development of such a medium, a procedure for the separation of red cells needed to be characterized. Red cells are most commonly separated by the serial and/or discontinuous centrifugation of dilute red cell suspensions, separations being determined primarily by cell density (Clark and Shohet, 1985). Hemoglobin constitutes the single greatest element of red cell protein. As cells mature hemoglobin content increases, overall cell density rises, and the heaviest cells are sedimented to the bottom of the centrifugation vessel. In addition, the maturation of trout erythrocytes is also characterized by increases in length and, to a lesser extent, width. Consequently, density sedimentation at unit gravity can be exploited for cell separations since this technique depends to a greater extent on cellular morphometry than does centrifugation (Miller and Phillips, 1969). In addition, cell losses due to mechanical perturbations and stress are reduced.

Once separated such cells can be examined to reveal ontogenetic variation. In this study, specific emphasis was placed on red cell morphometrics, including length, width, length:width ratio and cellular volumes. Developmental changes in hemoglobin system organization and isomorph abundance were

also examined through electrophoretic and staining techniques.

The goals of this study were therefore three in number:

(i) to develop a medium such that incubated red cells approximate a normal functional state,

(ii) to characterize a system for the separation of red cells into adult and juvenile fractions in terms of cell length, width and hemoglobin content, and,

(iii) to determine the hemoglobin isomorph system and relative abundancy of each component in adult and juvenile erythrocytes.

REVIEW OF LITERATURE

The literature on cardiovascular respiratory responses to increases in oxygen demand and/or availability is vast. Responses are not confined to gross systemic variations such as adjustments in blood circulation and gill perfusion. They also include alterations at the cellular and molecular levels. Rather than attempting to examine all aspects of the subject, this review has been confined to the events associated with the latter. Emphasis has therefore been given to the release of red cells from erythropoietic organs, their development of a system of hemoglobin isomorphs and the adaptive advantages such a system might confer.

These topics have been subdivided under the following general headings:

(1) the life history of the rainbow trout

(2) erythropoietic centers in teleosts

(3) blood cell formation in teleosts

(4) hemoglobin system organization in red blood cells, and

(5) hemoglobin switching during development.

Life history of the rainbow trout

The rainbow trout, Salmo gairdneri, is a moderately stenothermal freshwater teleost which is native to the western coast of North America from Alaska to California (Scott and Crossman, 1973). Introduction into Ontario occurred in 1904 with the planting of 20,000 eggs in the Sydenham River. This was extended under the sponsorship of the Ontario Department of Game

and Fisheries from 1918 onward. A favorite gamefish of anglers, rainbow trout are now found throughout most of eastern Canada and are permanent residents of lakes Superior, Huron and Ontario.

Rainbow trout occupy temperate well-oxygenated waters and have a temperature tolerance ranging from below 0° C to greater than 27° C with a final preferred temperature of 13° C (Garside and Tait, 1958). They are found in waters possessing a variety of acidic conditions and tolerate a pH range 5.8-9.5.

The lifespan of non-migratory rainbows ranges from 7-11 years while anadromous forms rarely exceed 9 years in age (McClane, 1974). Reproductive maturity is achieved as early as 1 year in males and as late as 6 years in females, the average age being 3-5 years in all types with males maturing a year younger than females. Adults migrate upstream in the spring to spawn in clear rapid waters and then return to lake regions. Adult size is attained in the second year at which time the fish average 30 cm in length. Size is often correlated with environmental conditions with larger fish being found in larger bodies of water. Average weight runs from 1-3 kg. Fish in excess of 8 kg having been reported in Ontario lakes while a high of 24 kg has been recorded in British Columbia (McClane, 1974).

Rainbow trout are carnivorous and normally feed on smaller fish such as perch and whitefish as well as various invertebrates including plankton, insects, snails and leeches (McKay, 1963).

Erythropoietic centers in teleosts

Hemopoietic centers in adult teleosts have been found in kidney (pro- and mesonephros), spleen, pancreas, liver, intes-

tinal tissue and gonad (Iuchi and Yamamoto, 1983). In the adult rainbow trout, however, this process is limited primarily to the kidney, head kidney and the spleen (Catton, 1951; Lane, 1979, Iuchi and Yamamoto, 1983).

Early studies by Downey (1909) on the ganoid kidney of the paddlefish, Polydon spathula, demonstrated the existence of a capillary network surrounding each kidney tubule. At certain points along the capillaries, injected India ink was observed to follow narrow channels which communicated with the intertubular tissue. A meshwork of peritubular channels and intertubular spaces was apparent, with strands of reticular cells providing structural support. No separation of erythrocytic or granulocytic precursor cells was reported with blood cells in various stages of development being found within this network. By comparison with fishes in which the kidney is not erythropoietically active the reticular stroma is often absent, suggesting that it may serve in the provision of blood stem cells (Catton, 1951).

The spleen of teleost fishes consists of parenchyma tissue containing blood vessels, red (erythrocytic) and white (lymphocytic) pulp, and macrophages surrounding which is a thin connective-tissue capsule. The organ is fed by small non-joining arteries which arise from the coeliac artery. These arteries branch out into the spleen, reduce to "capillary size" and are sheathed at the terminus to form ellipsoids. Rhythmic contraction of ellipsoids is thought to aid in blood movement through the spleen and has been suggested to have a hemoclastic function

in the removal and destruction of senescent and damaged cells from general circulation (Fange and Nilsson, 1985). Anastomosis of arterial and venous vessels, as observed in mammalian microcirculatory networks, is not found in the teleostean spleen and splenic circulation is described as being 'open'. The spleen is not ordinarily a major site of red cell formation in bony fishes. However, in relatively primitive salmonids, such as rainbow trout, erythrocyte stem cells are found within the splenic red pulp.

Studies on splenectomized animals have revealed only slight changes in the cellular components of circulating blood following spleen removal (Fange and Johansson-Sjobeck, 1975; Johansson-Sjobeck, 1979). This has prompted the conclusion that it acts primarily as a storage organ, providing a ready source of mature cells poised to enter circulation if or when needed. For example, Yamamoto et al (1980) found that hematocrit values increased sharply following prolonged stressful exercise in the form of continuous chase. This increase was due to release from the spleen.

Microdissection of the cod (Gadus morhua) spleen revealed innervation by anterior splanchnic nerves. The spleen was found to respond to both adrenaline/noradrenaline and acetylcholine addition (Nilsson and Grove, 1974). Further study employing 6-hydroxydopamine, a drug which specifically destroys adrenergic nerve terminals, led to a decline in catecholamine response values to levels typical of adrenergic denervation. However, acetylcholine response was also depressed to levels comparable to

those in animals in which cholinergic terminals had been removed (Winberg et al, 1981). It has been suggested that these responses confirm that the controlling nerve fibers are mixed in nature and have both adrenergic and cholinergic components. It therefore appears that under unfavorable conditions (e.g., prolonged physical exertion and hypoxia) erythrocytes are released through contraction of the spleen, the response being under nervous control and/or catecholamine stimulation.

The spleen also functions in the removal of senescent and damaged erythrocytes from general circulation. The mechanisms involved are not well understood although ellipsoid involvement as well as degradation by macrophages, have been implicated (Tischendorf, 1985). Movement through the narrow arterial channels would be expected to favor disruption of older, less resiliant cells while permitting passage of younger red cells.

Blood cell formation in teleosts

Jordon and Speidel (1924), advanced a polyphyletic hypothesis for the origin of red cells. This involved at least three precursor cell types, each of which gave rise to a cell lineage. The stem cells, termed 'lymphoid hemoblasts', were thought to develop from reticular cells of the reticulo-endothelial system. The three types of lymphoid hemoblasts defined differed from one another only in size, and were termed large, medium and small lymphoid hemoblasts. The large cells were thought to give rise to the granulocytic series (neutrophils, eosinophils and basophils), the medium-sized cells to

erythrocytes, and the smaller lymphoid hemoblasts to lymphocytes and thrombocytes (Fig. 1a).

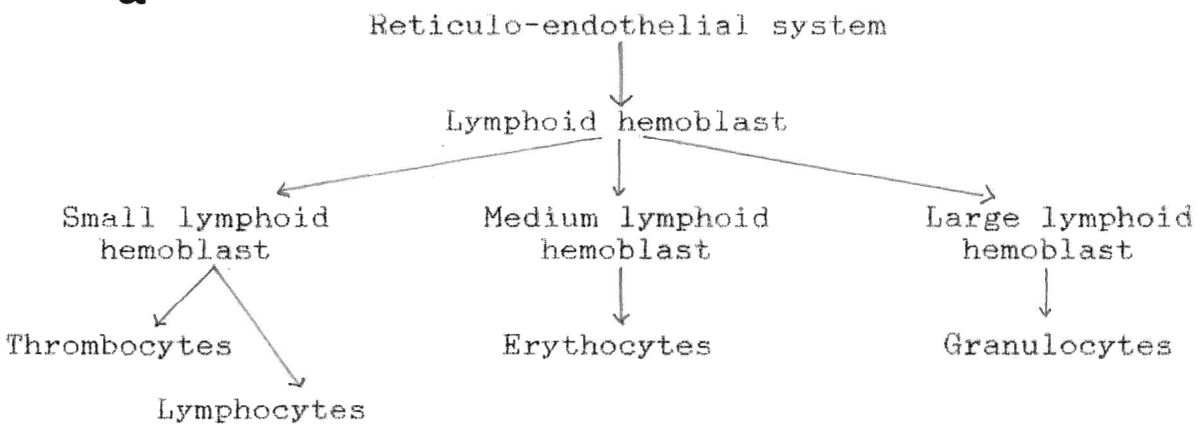
In subsequent studies, however, Duthie (1939) could distinguish only large and small lymphoid cell sizes. He renamed the large lymphoid hemoblasts 'granuloblasts', believing that they produced the granulocytic cell series, and used the name lymphoid hemoblast to denote the smaller lymphoid cells which divide into lymphocytes, thrombocytes, and erythrocytes. The large granuloblasts were thought to be the originating hemopoietic stem cells and to possess pluripotent capability. Duthie (1939) concluded that these cells become unipotent precursors of all cells including the small lymphoid hemoblasts. This view is described as the monophyletic theory of cell development (Fig. 1b).

Catton (1951) returned to the polyphyletic view of blood cell development following identification of two stem cell types. Again these differed only in size. Catton (1951) postulated that one stem cell type, the large lymphoid hemoblast, arose from the reticular cells of the reticulo-endothelial system and produced the granulytic series. In his view the small lymphoid hemoblasts arose from the endothelial cells of this system and produced all other blood cells (Fig. 1c).

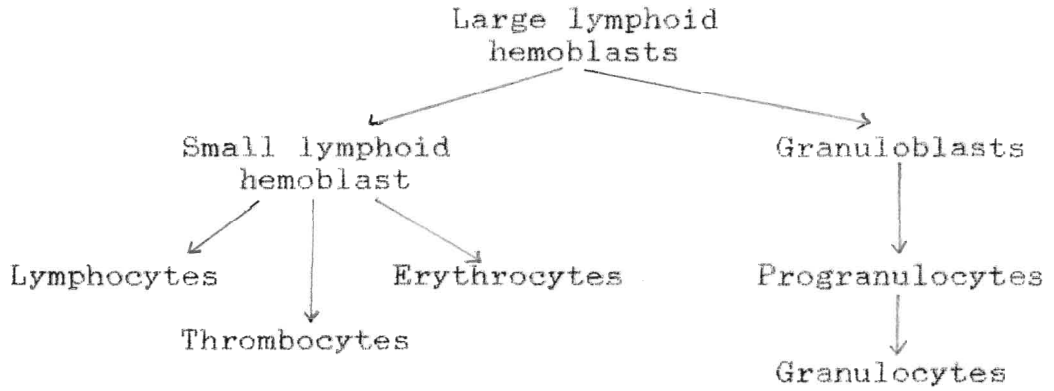
If the monophyletic view is true, then the single stem cell, which is inherently pluripotent, gives rise to all blood cell lineages and its developmental course is dependent on the environment in which it arises. Stem cells found in bone marrow, for example, would give rise to erythrocytes while those in lymph

Figure 1. Theorys of blood cell formation and development in fishes. (a) polyphyletic viewpoint of Jordon and Spiedel (1924). Hemoblast size is the determinant of eventual cell series. (b) monophyletic origin of red blood cells, proposed by Duthie (1939). All cell types stem from the large lymphoid hemoblasts which is pluripotent. (c) return to the polyphyletic view by Catton (1951). Two stem cell types arising from different components of the reticulo-endothelial system.

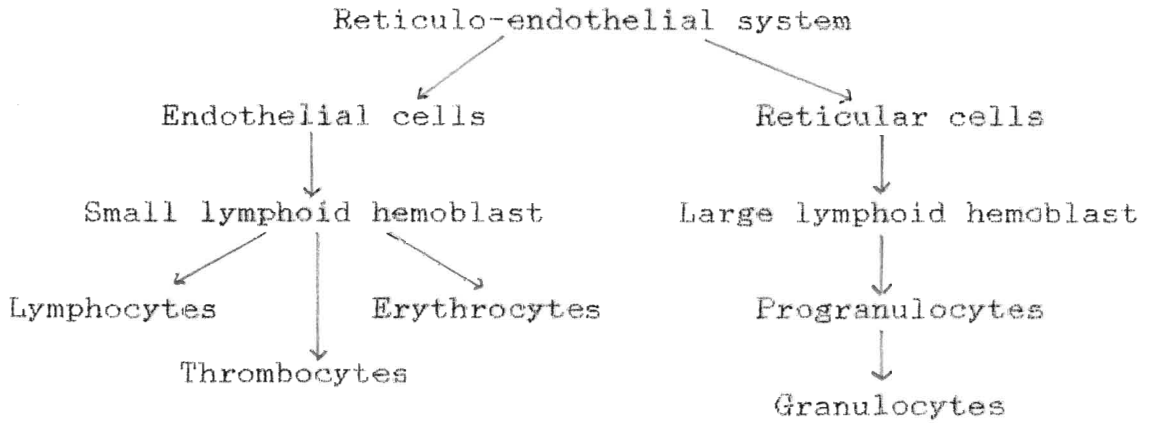
a


Reticulo-endothelial system
Lymphoid hemoblast
Small lymphoid hemoblast
Medium lymphoid hemoblast
Large lymphoid hemoblast
Thrombocytes
Erythocytes
Granulocytes
Lymphocytes

b


Large lymphoid hemoblasts
Small lymphoid hemoblast
Granuloblasts
Lymphocytes
Erythrocytes
Progranulocytes
Thrombocytes
Granulocytes

c


Reticulo-endothelial system
Endothelial cells
Reticular cells
Small lymphoid hemoblast
Large lymphoid hemoblast
Lymphocytes
Erythrocytes
Progranulocytes
Thrombocytes
Granulocytes

nodes would produce lymphocytes. The polyphyletic view, however, requires that two (or more) stem cell types exist, one producing lymphocytes only, and the other(s) producing all other blood cells. Each stem cell type has its own limited potentiality of development, and the environment exerts little influence on determination of cell type.

Catton (1951) referred to the work of Doan et al (1925) who found a segregation of sinuses in bone marrow which provided for the separation of developmental environments, and would therefore permit different cell types to form within the same general tissue. This finding provides support for the monophyletic viewpoint. However, as previously noted no such distinctiveness exists within the hemopoietically-active kidney of the teleost. Consequently, an explanation based on tissue specificity becomes more difficult and this can be viewed as indirect evidence which makes a polyphyletic view of blood cell formation in these animals somewhat more plausible.

Despite differences in the overall schemes of blood cell formation, common agreement is found in the development of erythrocytes. Blood cells originate from embryonic reticulo-endothelial tissue which has remained undifferentiated but retains the potential to become differentiated and specialize. Upon activation increases in nuclear and overall cell volume takes place and a stem cell is formed. The stem cell may then stay fixed within the tissue and release 'free' stem cells to circulation through mitosis, or it may break off and enter circulation. Development of large areas of condensed nuclear

chromatin marks the transition to a lymphoid hemoblast. The hemoblast ("medium-sized" by the Jordan and Speidel (1924) classification, "small" in Catton (1951)) gives rise within the erythropoietic tissue to an erythroblast. This cell type is characterized by continued condensation of chromatin, increase in the cytoplasm-to-nucleus ratio, a spherical to subovoid profile and increased size. Development to the reticulyte stage involves reduction in nuclear volume relative to that of cytoplasmic volume. It is at this stage that the maturing cells are usually released to peripheral circulation. While in circulation these cells, now regarded as immature erythrocytes, progress to their adult form (Catton, 1951). A shift from an ovoid to an elliptical profile is accompanied by an increase in the number of microtubules which comprise the marginal band system. This system has been suggested as producing cellular flattening (Cohen, 1978; Calarco et al, unpub. obs.). Decreases in the number of polyribosomes and other cytoplasmic organelles occur while NTP levels and hemoglobin content increase (Lane and Tharp, 1980; Lane et al, 1981). As the juvenile cells mature the number of mitochondria decrease and is correlated with the appearance of hemosomes (Brunner et al, 1977, 1980; Calarco et al, unpub. obs.). Studies demonstrating a close relationship between hemosomes and hemoglobin biosynthesis (Brunner et al, 1982, 1983b) suggest hemosomes to be the sites of incorporation of mitochondrially-produced heme into polysomally-generated globin chains (Brunner et al, 1983a).

Hemoglobin system organization in red blood cells

As in the case of mammals, teleosts are not restricted to having only a single form of hemoglobin. Consequently, they typically possess a number of electrophoretically-distinguishable hemoglobin isomorphs. In a comparison of some 300 vertebrate species DeSmet (1978) found multiple hemoglobin systems in all fishes examined; such systems were less common in mammals than in fish, amphibians, reptiles and birds. The number of hemoglobin components of a variety of fish species is presented in Table 1.

The potential advantage of complex systems is obvious. Fish inhabit environments which in many instances exhibit large fluctuations in temperature and/or dissovled oxygen content. If the components on the system are functionally heterogeneous the organism may have available one or more isoforms which acts optimally in oxygen uptake or oxygen release dependent upon the prevailing circumstances.

Hemoglobin polymorphism is thought to arise in a number of ways. In the adult human, for example, the principal hemoglobin is composed of two types of globin chains, α and β, combined in unlike pairs to form a tetrameric structure. In fish there must exist a larger number of globin types and/or more stable tetramer configurations. In the rainbow trout, three globins have been identified (Houston and Gray, 1985, unpub. obs). In goldfish isoform stability depends on temperature; two isoforms are found at $<10^{\circ}C$, three at temperatures $>12^{\circ}C$ (Houston and Rupert, 1976).

That fish hemoglobin can differ markedly in transport char-

Table 1. The number of hemoglobin isomorphs found in a number of fish species using starch gel electrophoresis (derived from Riggs, 1970).

Species of Fish	No. of Isomorphs
Carp (*Cyprinus carpio*)	4
Goldfish (*Carassius auratus*)	2-3
Rainbow trout (*Salmo gairdneri*)	11
Catfish (*Parositurus asotus*)	4
Loach (*Misguraus anguillicaudatus*)	11
Eel (*Anguilla japonica*)	4
Chum salmon (*Onchorynchus nerka*)	9
Goby (*Acontoogobius flavimanus*)	4
Opaleye (*Girella punctata*)	12
File fish (*Monocanthus cirrhifer*)	2
Red sea bream (*Chrysophrys major*)	8
Mackerel (*Scomber japonicus*)	3
Bigeye tuna (*Thunnus obesus*)	2
Yellowfin tuna (*Thunnus albacores*)	5
Albacore tuna (*Thunnus alalunga*)	6
Yellowtail (*Seriola quinqueradiata*)	7

acteristics has been well established. Examination of the properties of the 6 hemoglobin isomorphs of the lamprey, Petromyzon marinus, revealed differences in oxygenation properties, electrophoretic mobilities and amino acid composition (Rumen and Love, 1963; Rumen, 1966).

Intensive study of three of the major hemoglobin components of rainbow trout (referred to as Hb I, II and IV) by Brunori (1975) demonstrated each to be both structurally and functionally distinct. Hb I had a molecular weight of 16,300 daltons, while Hb II and IV had weights of 17,200 and 18,000 daltons respectively (Brunori, 1975). At pH's between 7.5 and 8.0, all exhibited cooperativity in oxygen binding. Over the range of 6.3 - 8.5, Hb I and Hb II showed no Bohr effects. Hb IV, however, displayed a decided right shift in the oxygen equilibrium curve with increasing acidity and a reduced oxygen carrying capacity. Between pH 6.0 - 6.5 the hemoglobins of some fishes can only be partially oxygen saturated. This phenomenon is known as the Root effect (Root, 1931) and permits the designation of hemoglobins as being Root or non-Root. Hb IV was found to be a Root hemoglobin while the lack of oxygen-linked effects of protons on Hb I and II designated them as being non-Root. Differences in thermal sensitivity were also observed. Hb I and II are relatively temperature-independent while Hb IV displayed proton-linked thermal responsiveness.

Thermoacclimation in fishes is sometimes coupled with at least modest increases in hemoglobin and hematocrit at higher temperature, i.e., increases in O_2-carrying capacity (DeWilde and

Houston, 1967; Houston and DeWilde, 1969; Cameron, 1970). Hemoglobin isomorph patterns are also modified during the acclimatory process. Acclimation of goldfish to temperature ranging from 5°C to 30°C resulted in a shift in the number of hemoglobin components from two at lower temperatures to three at higher levels (Faulkner and Houston, 1966; Houston and Cyr, 1974; Houston et al, 1976).

Changes in relative isomorph abundancies are more common than changes in the number of components. In the rainbow trout, for example, seven of nine electrophoretically-distinguishable hemoglobins, including the two major components, exhibited significant changes in actual concentration with temperature (Houston and Cyr, 1974). In a subsequent study of this species Tun (1985) found increases in hemoglobin and hematocrit with increases in temperature, exposure to hypoxia and decreases in daylight hours. Of the twelve isomorphs observed, the two most abundant cathodal hemoglobins increased with increasing water temperature, whiile the most abundant anodal component was not thermally influenced. Almost all components were found to increase under conditions of hypoxia.

Comparable changes in isoform concentrations have also been observed as accompaniments of thermal acclimation in bullhead (Ictalurus nebulosus, Grigg, 1969), sucker (Castostamus commersoni), pumpkinseed (Lepomis gibbosus), carp (Cyprinus carpio), goldfish (Carassius auratus) and the natural hybrid of the latter species (Houston, 1980).

Polymorphic hemoglobins can also be distinguished in terms

of their responses to organophosphate and other modulators of hemoglobin-O_2 affininty. The hemoglobins of the ciclid, Cichlasoma cyanoguttatum and the freshwater catfish, Pimelodus maculatus, displayed little sensitivity to changes in pH or organophosphate concentration (Gillen and Riggs, 1971; Reischl, 1977). The carp, Cyprinus carpio, however, possesses at least two major hemoglobin isomorphs sensitive to both factors (Noble et al, 1970; Weber and Lykkeboe, 1978). A number of species including the chum salmon, Oncorhynchus keta (Hashimoto et al, 1960), rainbow trout, Salmo gairdneri (Binotti et al, 1971), and the white sucker Catostomus commersoni (Powers and Edmundson, 1972) have hemoglobins displaying both responses.

Hemoglobin Switching During Development

Hemoglobin switching during development has been observed in all vertebrate classes. Larval red cells, newly released to circulation, display a different hemoglobin system organization from that of adult counterparts. As the animal matures, a shift occurs from a particular set of hemoglobins to another; each set is distinct and provides the organism with different oxygen transport capabilities.

In the coho salmon, Oncorhynchus kisutch, up to eighteen different isomorphs can be electrophoretically distinguished. Only three of these are present throughout the development of the fish from embryo to adult. The remaining fifteen isomorphs characterize the progression from freshwater larva and fry through pre-migrant smolt to seawater-adapted adult by their presence or absence and by their relative proportions (Giles and

Vanitone, 1976). Comparison of fry and adult hemoglobins demonstrated that those of fry had a high oxygen affinity, a large Bohr shift and a marked sensitivity to nucleotide triphosphates. Adult hemoglobins, on the other hand, exhibited a moderate oxygen affinity, a small Bohr shift and were not greatly influenced by organophosphate modulators (Giles and Randall, 1980). In an examination of larval and adult red cells of the rainbow trout Iuchi (1973a) found the less mature form to have hemoglobins characterized by higher oxygen affinity and a lower Bohr effect than red cells of adults.

Functional differences between hemoglobin isoforms are usually associated with differences in globin chain composition. In man the principal hemoglobin synthesized in fetal life is fetal hemoglobin ($\alpha_2\gamma_2$) and that in adult life is adult hemoglobin ($\alpha_2\beta_2$). Although both are different, they share a common α polypeptide chain, the switch representing a shift from γ to β chain synthesis (Rowley et al, 1979). A similar situation exists in mouse and chicken where fetal and adult hemoglobins have a common peptide chain (Ingram, 1972). In the bullfrog, Rana catesbeiana, there are no common chains (Maniatus and Ingram, 1971b).

Developmental shifts in hemoglobin organization can be brought about in two ways. Alteration in the globin synthesizing machinery within individual cells of a single lineage can occur; genes responsible for production of one set of globins being turned off while those leading to production of others is stimulated. Under such circumstances, cells in the transition

stage should exhibit both isomorph patterns. Increases in one system over the other would then occur as the animal matured.

The alternative process involves the switching, not of hemoglobin production from one type to another, but a switching of the lineages involved in red cell formation. Thus a shift from erythropoietic tissues releasing cells possessing the capacity to form a particular isomorph pattern to others whose cells can synthesize a different set of hemoglobins will also account for the switching phenomenon. Under such circumstances any given cell could express only a single pattern of hemoglobins. With the switch from fetal to adult erythropoietic systems a new population of cells would arise producing exclusively adult hemoglobin types. These two mechanisms are not, of course, mutually exclusive; a shift in erythropoietic sites does not necessarily mean a shift in hemoglobin complement. It is possible that both occur but the transition from larval to adult isomorph patterns reflects intracellular events independent of erythropoietic site.

As the following studies reveal, evidence can be adduced to support the latter mechanism. In Rana catesbeiana the primary sites of red cell formation are liver, spleen, kidney and bone marrow. In addition, new cells can be formed in circulation. In premetamorphic and metamorphic tadpoles the liver is the major organ of erythropoiesis; in the adult this is in bone marrow (Maniatus and Ingram, 1971a). Immunological studies have revealed that larval and adult hemoglobins are not present in the same cell at the same time (Maniatus and Ingram, 1971b), such

observations lending support to the second hypothesis of hemoglobin switching.

Ingram (1972) examined the hemoglobins of chick embryo and adult erythrocytes. Embryo red cells arise in the mesenchymal tissue after 24 hours of incubation, and hemoglobin can be detected approximately 12 hours later. With the development of a circulatory system after 48-60 hours of incubation, red cells are released and function in oxygen transport. Adult cells begin to appear in circulation after Day 7 although some embryonic red cells are formed after day 13. Adult cells arise initially from yolksac tissue and later from bone marrow. As noted previously, the hemoglobin isomorph pattern is different in embryonic and adult erythrocytes and the change is correlated with a shift in eythropoietic site.

In man, red cells shift in hemoglobin complement from a state consisting primarily of Hb F in the fetus to a predominance of Hb A and, to a lesser extent, Hb A_2 in the adult. Unlike the bullfrog, but similar to the South African clawed toad, Xenopus laevis (Jurd and Maclean, 1970), both adult and fetal hemoglobins are found within the same circulating erythrocytes (Dover and Boyer, 1980); Hb F, however, represents only 0.2% of the total hemoglobin complement of circulating red cells. The shift in predominance of hemoglobin isomorph (Hb F to Hb A) may be linked to cell origin, fetal erythrocytes being primarily derived from the liver while the major site of erythropoiesis in adults is bone marrow.

In the rainbow trout, larval erythrocytes (Ery L) are round

and disc-like (Iuchi, 1973b) and are formed in the intraembryonic "intermediate cell mass" as well as in the extra-embryonic blood islands of the yolk sac. These cells persist for 7-12 days post-fertilization. Their production ceases by the 15th day or 5 days prior to hatching. Replacement by small, elliptical, disc-shaped immature adult cells (Ery ImA) occurs when erythrocyte formation shifts to the kidney and spleen. Maturation to the adult stage (Ery A) ensues in circulation and involves the changes previously noted (pg. 13). Immunofluorescence studies with FITC-antibody probe (sensitive to hemoglobins of Ery ImA and Ery A) indicated the production of these cells from erythropoietic organs one day prior to hatch. The inability of the antibody to detect larval hemoglobins was interpreted as different hemoglobin complements in the two cell population types (Ery L and Ery ImA/A). Hemoglobin switching was correlated with the shift in erythropoietic site during development, and was thought to reflect red cell replacement rather than alteration of hemoglobin synthesizing machinery within the cell (Iuchi and Yamamoto, 1983). Therefore, while the development of new erythropoietic sites have been found to produce cells possessing a different hemoglobin isomorph pattern than previously existing cells, it has not been demonstrated that cells already in circulation are capable of altering their hemoglobin synthesizing machinery to produce new isomorph components.

MATERIALS AND METHODS

Origin and maintenance of experimental animals

Yearling rainbow trout (Salmo gairdneri) were purchased from a commercial supplier (Goossen's Trout Farm, Otterville, Ontario). Fish ranged in fork length from 24.5 cm to 35.0 cm ($\bar{X}$ = 28.9 cm), and in weight from 163.3 g to 554.4 g ($\bar{X}$= 298.5 g).

Upon arrival in the laboratory animals were examined for disease symptoms and vigour, and evenly distributed between two recirculating double-walled 500 L fiberglass tanks (LS-700, Frigid Units Inc., Toledo, Ohio). Each tank was equipped with a Cul-Brook water dechlorination/filtration system (Culligan International Company, Mississauga, Ontario). The circulation time of these tanks is 1.5 minutes (Smeda, 1979), ensuring uniformity of water quality parameters such as temperature, pH and dissolved oxygen. Water temperature was controlled to 14°C $\pm$ 1°C by opposition of heating and cooling units whose operations were governed by thermoregulators of local design and construction. Each tank was equipped by circulation units which, by splash aeration, provided dissolved oxygen contents at, or near, saturation levels (> 85%, i.e. 8.8 ppm). Photoperiod hoods maintained a 12:12 light:dark photoperiod regime with light intensities ranging from 90 to 120 lux at the water surface.

Animals were fed once daily ad lib. on Purina Trout Chow. Fecal matter and excess food was removed by siphoning at least twice a week and filter screens were changed as needed.

Sampling procedure

To avoid possible complications from chemical anesthetic effects, fish were stunned by a blow to the head before sampling. Mixed blood was drawn from the caudal vessels immediately behind the anal fin into syringes previously rinsed with chilled ammonium heparin (Sigma Chemical Co., St. Louis, Missouri, 50,000 units). Subsequent analytical and separation procedures are outlined in the following pages.

Hematocrit (Hct, %)

Freshly-sampled whole blood was drawn in microcapillary tubes. These were closed with "Critoseal", a commonly-used tube sealant, and centrifuged at 7000 rpm for 5 minutes at room temperature (20-25 C). Hematocrit values were determined on an Adams Micro-Hematocrit Reader (Clay Adams, Parsippany, NJ).

Hemoglobin (Hb, $g \cdot dL^{-1}$)

Two different techniques were employed in the determination of hemoglobin content; the alkaline hematin method and cyano-methemoglobin procedure.

The alkaline hematin method, as outlined by Anthony (1961), was initially used. In this procedure iron in the Fe (II) state is oxidized to Fe (III). Twenty microliters of packed cells were added to 5.0 mL of 0.1N NaOH and placed in a boiling water bath for 4 minutes. After cooling, absorbance values were determined at 560 nm using a Bausch & Lomb Spectronic 100. The technique has proven to be accurate in cases of packed cells but displays considerable scatter when whole blood is used (Anthony, 1961).

Discrepancies are thought to result from non-Hb iron present due to erythrocyte breakdown within the bloodstream. As the hemoglobin from the red cell is released it becomes denatured and the iron is released to plasma. A second procedure was thus employed in studies requiring hemoglobin values for whole blood, i.e. the cyanomethemoglobin method.

The cyanmethemoglobin determination uses a reagent containing $K_3Fe(CN)_6$, KCN, KH_2PO_4 and Nonidet-P40 (a detergent). The cells lyse and the hemoglobin is oxidized by ferricyanide to methemoglobin. This compound is then converted to the highly stable cyanmethemoglobin derivative by cyanide. The reaction rate proceeds quickly if a phosphate buffer is used to maintain the pH at optimal levels (7.0-7.4). Twenty microliters of whole blood were added to 5.0 mL of the cyanide reagent, vortexed for 1 minute to facilitate hemolysis and allowed to stand 5 minutes for reaction completion. Again, optical densities were determined spectrophotometrically at 540 nm against bracketing standards.

In the incubation studies subsequently described, samples were drawn from incubation flasks and first centrifuged. Hemoglobin determinations were then carried out on the packed cells rather than whole blood. In all instances stabilized uniform human hemoglobin preparations (Boehringer Mannheim Diagnostics, Inc., Indianapolis, Indiana) were used as standards.

Red Cell Incubation Procedures

The media employed in this study was developed through a modification of the original Cortland saline formulation

described by Wolf (1963). In its original form this widely-used saline is unsatisfactory in maintainance of red cell volume and ionic integrity (Greaney and Powers, 1978; Houston et al, 1985). The composition of Cortland-3, the final medium developed, is summarized in Table 2. Cortland-2 represents a modification prior to the final form; the principal differences between the two involve the presence or absence of norepinephrine and a slight difference in calcium levels. The pH of the medium was 7.9, consistent with that of rainbow trout blood kept under the previously described holding conditions (Heisler, 1984).

In preparation of erythrocyte cultures, freshly drawn blood was added to 10 mL of phosphate-buffered saline (PBS, 0.9%) and the cells repelleted through mild centrifugation (500g, 5 min, 14°C). The supernatant was discarded and the cells washed first in a second 10 mL aliquot of PBS and subsequently in 5 mL PBS + 5 mL Cortland-2 or -3 depending upon the trial conditions. Cells were repelleted between washings. After a final centrifugation the supernatant was discarded, and the pellet resuspended in 10 mL of the appropriate medium. The cell suspension was then added to 50 mL of medium in 250 mL flasks. Flasks were wrapped in aluminum foil to prevent exposure to light as norepinephrine is photosensitive when in solution. Cultures were constantly swirled in a gyrorotary shaker bath at 14°C ± 0.1°C and samples were drawn after 0, 2, 8 and 24 hours of incubation.

Water content determinations

Following sample removal, cells were pelleted by centri-

Table **2**. Medium composition of unmodified Cortland saline, Cortland-2 and Cortland-3. All values are listed in mmoles/L.

Component	Unmodified Cortland	Cortland-2	Cortland-3
NaCl	124.06	124.06	124.06
$MgSO_4 \cdot 7H_2O$	0.93	0.93	0.93
$NaH_2PO_4 \cdot 2H_2O$	2.63	2.63	2.63
$CaCl_2$	1.56	1.00	0.88
KCl	5.10	3.35	3.35
$NaHCO_3$	11.90	29.76	29.76
D,Glucose	5.56	2.78	2.78
Hepes buffer (Na salt)	---	9.15	9.15
Pyruvate (Na salt)	---	4.00	4.00
Heparin (NH_4 salt)	---	0.005% w/v	0.005% w/v
Bovine Serum Albumin	---	0.030% w/v	0.030% w/v
Norepinephrine (arterenol HCl)	---	---	0.005

fugation at 1100 rpm for 5 minutes at 14°C and the supernatant removed and retained for analysis. Ten microliters samples of packed cells were drawn into preweighed micropipettes and cell weight determined. Pipette and contents were then dried for 24 hours at 70°C followed by an additional 48 hours at 103°C. Dry weights were determined and the water content estimated by difference.

Chloride determinations

Chloride determinations of red blood cells and plasma were carried out by electrometric titration involving chloride precipitation with silver ions as silver chloride (AgCl). The solvent employed was a solution of 0.1 N HNO_3 and 10 % glacial CH_3COOH. Plasma samples (20 µL) were added to 2.0 mL of solvent just prior to titration. Packed red cell samples (10 µL) were pipetted into the solvent (2.0 mL) the night before and allowed to digest in the refrigerator at 2-4°C for at least 12 hours prior to use in order to ensure complete hemolysis. Immediately prior to analysis, 2 drops of 0.62 % gelatin solution (60:1:1 gelatin:thymol blue:thymol in distilled water) were added to each sample. Sodium chloride solution (4.00 mM NaCl) and Versatol (General Diagnostics, Morris Plains, New Jersey) were used as standards. Versatol is an artificial human serum having a chloride concentration of 103 mEq per Liter. Distilled water served as a blank.

Determinations were carried out using the Buchler-Cotlove Chloridometer, Model 4-2000 (Buchler Instruments Inc., Fort Lee, New Jersey). In this system silver ions are generated by passing

a constant direct current through a pair of silver electrodes. The Ag^+ produced by this coulometric circuit are released into the sample solution containing the Cl^- and AgCl precipitation occurs. A timing mechanism is also activated by initiation of Ag^+ generation. Once the titration end-point is reached, i.e. all chloride ions have precipitated out of solution, the free Ag^+ concentration increases, causing a rising current to flow through a pair of silver indicator electrodes (the amperometric circuit). This current increase activates a meter-relay within the system and stops the timer. As rate of Ag^+ release into the analyte is constant, and Ag^+ and Cl^- combine in a 1:1 ratio, the amount of Cl^- in the sample is directly proportional to the elapsed time.

$$Ag^+ (aq) + Cl^- (aq) \rightarrow AgCl (s)$$

Titration endpoint does not coincide exactly with the stopping of the timer since current must be built up by excess Ag^+ to trip the meter-relay. The distilled water blank is therefore used to determine the time required for current buildup and is then subtracted from the final elapsed time. Thus,

$$Cl^- \text{ conc'n (mM)} = \frac{Ts - Tb}{Tstd - Tb} \times \text{Std Conc'n}$$

where Ts = titration time for sample (sec)
Tb = titration time for blank (sec)
Tstd = titration time for standard (sec)
Std Conc'n = 3.9989 mM NaCl

Cation determinations

Cation determinations (K^+, Mg^{+2}, Ca^{+2}) were conducted by atomic absorption spectrophotometry. A Perkin-Elmer model 372 ASS was used in the absorption mode for Ca^{+2} and Mg^{+2} and in the

emission mode for K^+. Sodium is present in appreciable amounts in trout erythrocytes and, in atomic absorption analysis, causes positive interference in K^+ determinations; K^+ levels are overestimated as a consequence. Emission mode, however, produces increased sensitivity and reduced interference and was employed in this instance. The standards also contained both Na and K to ensure that neither calibration nor sample values differed in interference levels. Mg^{+2} and Ca^{+2} concentrations, however, were better determined using the absorption mode of spectroscopy.

Flask samples were centrifuged at 2500 g for 10 minutes and the supernatant removed for later analysis. Plasma and packed cell samples were stored at -76°C until used. Earlier studies (Houston and Smeda, 1979) indicate that no compositional changes occur under these conditions for at least six months.

Fifty microliter samples of packed cells were pipetted into 5.0 mL of distilled water and allowed to hemolyze overnight in the refrigerator (2-4°C). Following this, 5.0 mL of 15.29 g/L $SrCl_2 \cdot 6H_2O$ was added. Membrane debris was removed by centrifugation at 3200 rpm for 15 minutes at 14°C. Plasma samples were treated in much the same way except that 100 µL of plasma (or medium) was added to 5.0 mL of 7.76 g/L $SrCl_2 \cdot 6H_2O$. In both instances a final background concentration of 7.61 g/L $SrCl_2 6H_2O$ resulted.

Combinations of single-element BDH standards were used to construct bracketing calibration curves and the spectrophotometer was blanked with 7.61 g/L $SrCl_2 \cdot 6H_2O$. Different $SrCl_2$ concentrations were used in sample and standard preparation to ensure a

final background concentration of 7.61 g/L $SrCl_2 \cdot 6H_2O$ in each case. As with chloride determinations, Versatol was employed as a reference.

The "Trapped Plasma" Factor

In estimating cellular ion concentrations consideration must be given to plasma trapped in the interstices of the packed cell column. Houston and Smeda (1979) determined that "trapped plasma" represented 2.82% of the packed cell total, a value in good agreement with the estimate of Catlett and Millich (1976) for goldfish blood (3%). In instances where plasma ion levels exceed those of cell concentrations, estimated cellular values will be erroneously high unless correction is made. Thus,

$$\underset{(mM \cdot L^{-1},\ pc)}{\text{Corrected ion level}} = \frac{E - (P \times 0.0282)}{0.9718}$$

where E = uncorrected erythrocyte ion level ($mM \cdot L^{-1}$, pc)
P = plasma ion level ($mM \cdot L^{-1}$, medium)
0.0282 = % of total from trapped plasma
0.9718 = % of total from packed cells (1 - 0.0282)

The values reported from this calculation are means for the entire aqueous phase of the cell. In this manner, problems associated with intracellular compartmentalization, the effects of nonspecific interionic attraction and specific ion binding are avoided.

Packed cell hemoglobin and water contents were similarly treated, operating under the assumption that trapped plasma constitutes 2.82% of the total volume used in determinations.

Separation of Red Cells by Velocity Sedimentation at Unit Gravity

In preparation for cell separations, freshly drawn blood was

added to 10 mL of Cortland-3 saline containing 0.3% Bovine Serum Albumin (BSA) at 14°C. Cells were then pelleted by mild centrifugation (500 g, 5 minutes, room temperature). The supernatant and "buffy layer" of leucocytes were discarded, and the pellet resuspended by gentle addition of 25-30 mL of Cortland-3 + 0.3% BSA.

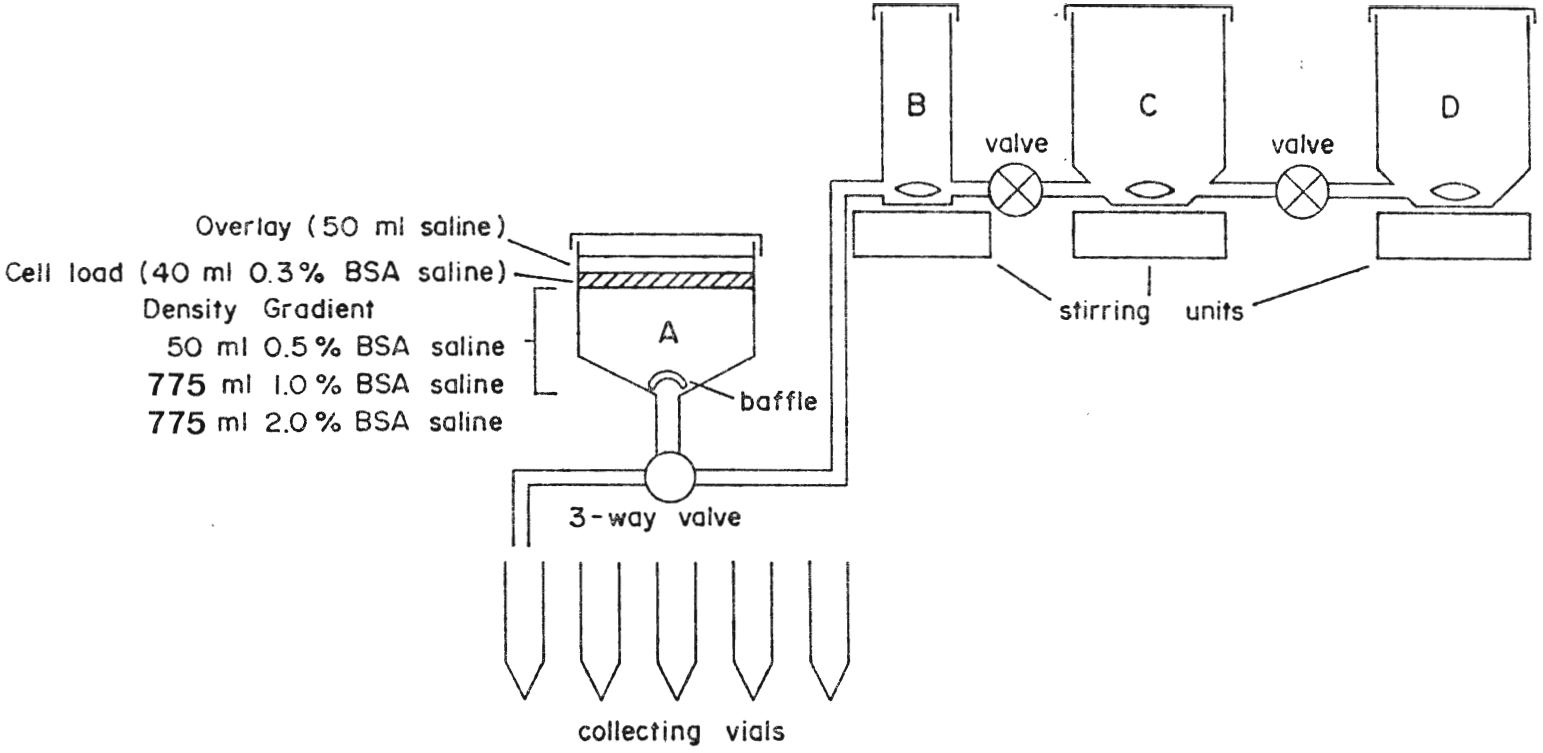
Since there is an upper limit to the number of red cells which can be added to the separation system, red cell counts were next made to determine the number of erythrocytes per milliliter of suspension. Ten microliters of red cell suspension were added to 1.0 mL of Cortland-3 + 0.3% BSA and mixed by gentle inversion. A drop of this solution was placed on an Improved Neubauer Counting Chamber (Hausser Scientific, Blue Bell, Pennsylvania) and viewed with a Leitz Laborlux II Microscope at 100x magnification. The number of red cells was determined as follows:

$$\#\ \text{red cells/mL} = \frac{\#\ \text{counted}}{\text{Chamber volume (mL)}} \times \text{dilution factor}$$

$$= \frac{\#\ \text{counted}}{0.1 \times 0.1 \times 0.001} \times 101$$

Separations were carried out with a Johns/Miller "Sta-put" Apparatus (Johns Scientific, Toronto, Ontario). The system consists of three loading chambers and a single collecting chamber (Figure 2). The separation procedure is based on differences in cell sedimentation rates under unit gravity which arise primarily as a result of differences in cell size, shape and density. The cells were loaded into the system and allowed to fall through a non-linear gradient of 0.3 to 2.0 % BSA for 3 hours. This gradient prevents mixing of adjacent layers during

Figure 2. The John's/Miller Sta-put Apparatus. The system separates red cells by sedimentation under unit gravity through a discontinuous medium. Chamber A is the collecting chamber; Chambers B, C and D are loading chambers.


B
C
D
valve
valve
Overlay (50 ml saline)
Cell load (40 ml 0.3% BSA saline)
Density Gradient
50 ml 0.5% BSA saline
775 ml 1.0% BSA saline
775 ml 2.0% BSA saline
A
baffle
stirring units
3-way valve
collecting vials

loading and unloading and stabilizes the system against thermal convection events. Convection causes "streaming" to occur and leads to streamers of 1 cm or more in length which extend from the cell layer into the layers below (Miller and Phillips, 1969). Streaming can also be induced by overloading the system with cells. A "streaming limit" or maximum cell load exists above which streamers are likely to occur. In the Sta-put system the streaming limit is 1.5×10^9 cells making prior cell counts essential. The entire procedure is carried out at 4°C to minimize thermal convection and to increase cell viability.

Fifty milliliters of Cortland-3 were first loaded into Chamber B (Fig. 2) and allowed to flow into the collecting chamber (Chamber A). All bubbles were removed from the tubing and from beneath the baffle. Flow was stopped just prior to air entry into the now air-free tubing and 40 mL of the cell suspension in Cortland-3 + 0.3% BSA was poured into the loading chamber (Chamber B). The magnetic stirrers were turned on to aid in cell loading. The total number of red cells present in the 40 mL load was 1.5×10^9 cells, i.e., the streaming limit. Once the cells were loaded the underlay solutions were added. Fifty milliliters of Cortland-3 + 0.5% BSA were added to the loading chamber (B) and 775 mL of media + 1.0% BSA and 775 mL of media + 2.0% BSA were added to chambers C and D respectively. The clamps between chambers C and D and between B and C were opened. The solutions came into equilibrium, and the stopcock was opened to permit them to flow into the collecting chamber. After all solutions had entered chamber A, the stirring bars were

turned off. Three hours after the erythrocytes had initially entered the collecting chamber, fractionation was started. The first 350 mL wre removed and discarded as this represented the cone volume. The remainder was drained off and collected into marked 40 mL vials. Thirty-two vials were typically obtained through this procedure.

Vials were centrifuged in an IEF Refrigerated Centrifuge at 4°C for 5 minutes at 1100 rpm. Supernatants were removed and cells gently resuspended by swirling with a small volume of media. Vial contents were quantitatively transferred such that the cells in vials 1, 2 and 3 were contained in a single vial, "A". Vials 1-3 represent the first cell suspensions recovered from the Sta-put, i.e., cells which had dropped the greatest distance. This procedure was continued until 10 vials labelled "A"-"J" had been collected. The remaining vials (31, 32, residual) were discarded as these fractions contained almost no cells. Any found in them were usually a heterogeneous group of adherent cells picked up as the last of the solution flowed down the funnel at the base of the collecting chamber (It has been shown that cells sometimes adhere to the walls of the chamber and are only released when the last of the solution is removed). They do not represent cells which have sedimented in a "normal" fashion.

Red cell counts were made for each vial and hemoglobin analyses performed. The suspension was again spun down (1100 rpm, 5 minutes) and the supernatant removed. Remaining fluid was eliminated by absorption with a cotton swab.

In other series of Sta-put separations, the recovered cells were grouped into vials A-J and the pellet stored in the Ultra-cold at -76°C for subsequent electrophoretic analysis.

Red cell morphology

Microphotographs of cells from vials A-J were taken at 400x power using a Leitz Laborlux II Microscope with a Wilde MPS 15/12 Camera attachment. Photographs under similar conditions were also made of a calibrated micrometer. Cell dimensions then were determined by caliper measurements of photographs. These were subsequently calculated in u using a magnification factor acquired through comparison of micrometer pictures with actual scale dimensions.

Cell volumes were estimated by consideration of the cell as a prolate spheroid, using the formula:

$$\text{Cell Volume } (u^3) = (4/3)(n)(L/2)(W/2)^2$$

where L = cell length (u)
W = cell width (u)

Electrophoresis of Separated Red Cells

Stored packed red cells were washed three times in 0.85 % NaCl and hemolyzed in two volumes of distilled water. These were then vortexed for 2 minutes and centrifuged at 3400 rpm for 10 minutes at room temperature, the hemolysates were removed and the membrane pellet discarded. Hemoglobin isomorphs were stabilized in the carboxy configuration by bubbling carbon monoxide through hemolysate samples for 3 minutes.

Electrophoresis was carried out on cellulose acetate strips

(Titan III-H, Helena Laboratories). A Tris-EDTA-boric acid buffer was used (Supre-Heme, pH 8.2-8.6, 0.025 ionic strength). After having soaked the plates (strips) for 5 minutes in the buffer, hemolysate samples were applied. Separations were carried out at 400V for 20 minutes at 4°C.

Following separation, strips were stained for 3 minutes in a protein stain, Ponceau-S, and washed in 5 % acetic acid (3 times, 2 minutes each) and methanol (2 times, 2 minutes each). Finally, strips were transparentized by soaking in Clear-Aid, a commercial methanol-acetic acid mixture. Strips were air-dried for 3 minutes and then oven-dried for 5 minutes at 70-75°C.

Hemoglobin isomorph mobilities were compared with that of HbA1, a human hemoglobin standard (AA2 hemocontrol, Helena Laboratories) electrophoresed concurrently with hemolysate samples in all instances. Mobility measurements were made with vernier calipers (Sargent-Welch Scientific Company, Skokie, Illinois) and Rf values calculated by the equation:

$$Rf = \frac{\text{mig Hbi (cm)}}{\text{mig HbA (cm)}}$$

where Rf = relative fluidity
mig Hbi = migration distance of Hb isomorph
mig HbA = migration distance of HbA1 standard

Relative isomorph abundancies were determined by using an "Auto-Scanner Flur-Vis" densitometer (Helena Laboratories, Beaumont, Texas). This was equipped with a 525 nm filter since the Ponceau S dye-protein complex has maximal absorption at this wavelength. Abundancies are determined by differences between the amount of light emitted at the source and the amount that

reaches the detector head.

Statistical Analysis

Mean, standard error (SE) and 95% confidence intervals (95% CI) were calculated for all samples. Kolgomorov-Smirnov tests found all red cell culture results to be normally distributed. Samples were tested for significant differences in ion levels, hemoglobin concentration, and water content over the 24-hour incubation period using Student's t-tests at a 0.05 level of significance.

Relative mobility values of all bands and hemoglobin abundancy data of major bands, i.e. those bands found in all instances, were tested for normal distributions using Kolgomorov-Smirnov tests at the 0.05 level of significance. Dependent upon outcome, testing for differences between groups was made parametrically or non-parametrically using, respectively, analysis of variance (ANOVA) or the Kruskal-Wallis one-way analysis of ranks.

RESULTS

A. Red Cell Incubation Studies

1. Medium Composition

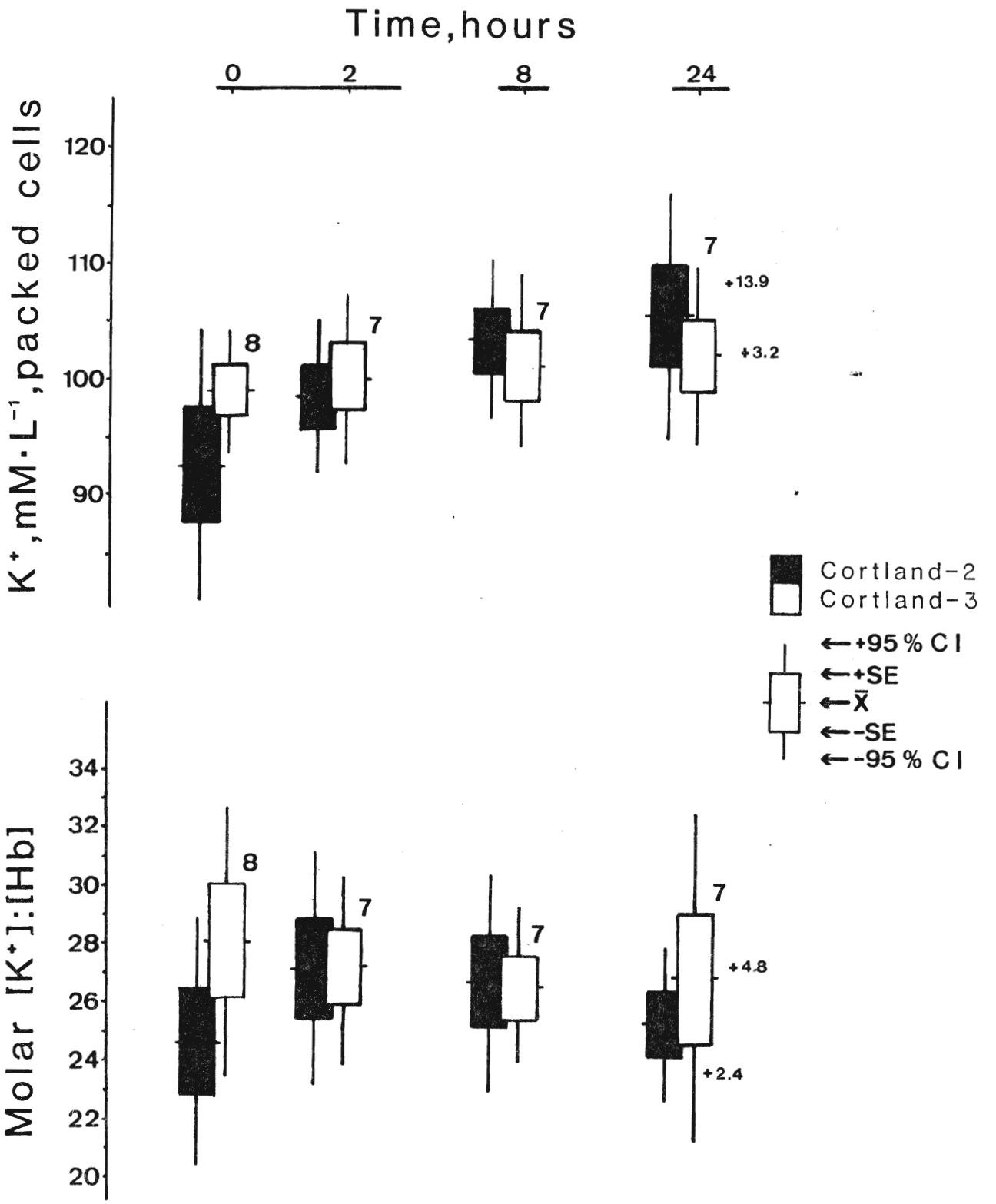
Use of unmodified Cortland saline leads to marked decreases in nucleotide triphosphate (NTP) levels, water content and cell volume as well as major changes in ionic content. Through reduction in calcium and chloride levels, increase in buffering capacities and pyruvate supplementation (Cortland-2, Table 2), NTP values can be stabilized (Houston et al, 1985). Clotting was avoided through addition of small amounts of the anticoagulant, ammonium heparin. Despite these modifications, however, increases in intracellular magnesium levels persisted, as did losses of calcium, potassium and chloride. Increases in packed cell hemoglobin proved to be well correlated with concommitant decreases in cell water content and hematocrit (i.e. red cell volume). Since no significant changes in overall hemoglobin content were observed, these changes almost certainly stem from the increased number of smaller red cells per unit sample volume.

Final modifications to the medium (Cortland-3) included a further decrease in calcium and addition of a physiologically-realistic level of noradrenaline, a hormone implicated in potassium ion regulation by Bourne and Cossins (1982).

2. Ion Determinations

Studies using Cortland-2 as the incubating medium showed a non-significant increase of 13.9% in potassium ion concentration over the 24-hour incubation period (Fig. 3, Appendices I, IIa)

Figure 3. Variations in potassium ion levels and molar ion:hemoglobin ratio during a 24 hour incubation period in either Cortland-2 (closed boxes, without hormone) or Cortland-3 (open boxes, with hormone) saline. The number above each box-and-whisker diagram represents the n value for each while that in small print to the right is the percent change in mean 24 hour value from mean 0 hour (original) level. Flasks were incubated at 14 C $\pm$ 0.1 C.


Time,hours
0
2
8
24
K+,mM·L-1,packed cells
120
110
100
90
8
7
7
7
+13.9
+3.2
Cortland-2
Cortland-3
←+95 % CI
←+SE
←X̄
←-SE
←-95 % CI
Molar [K+]:[Hb]
34
32
30
28
26
24
22
20
8
7
7
7
+4.8
+2.4

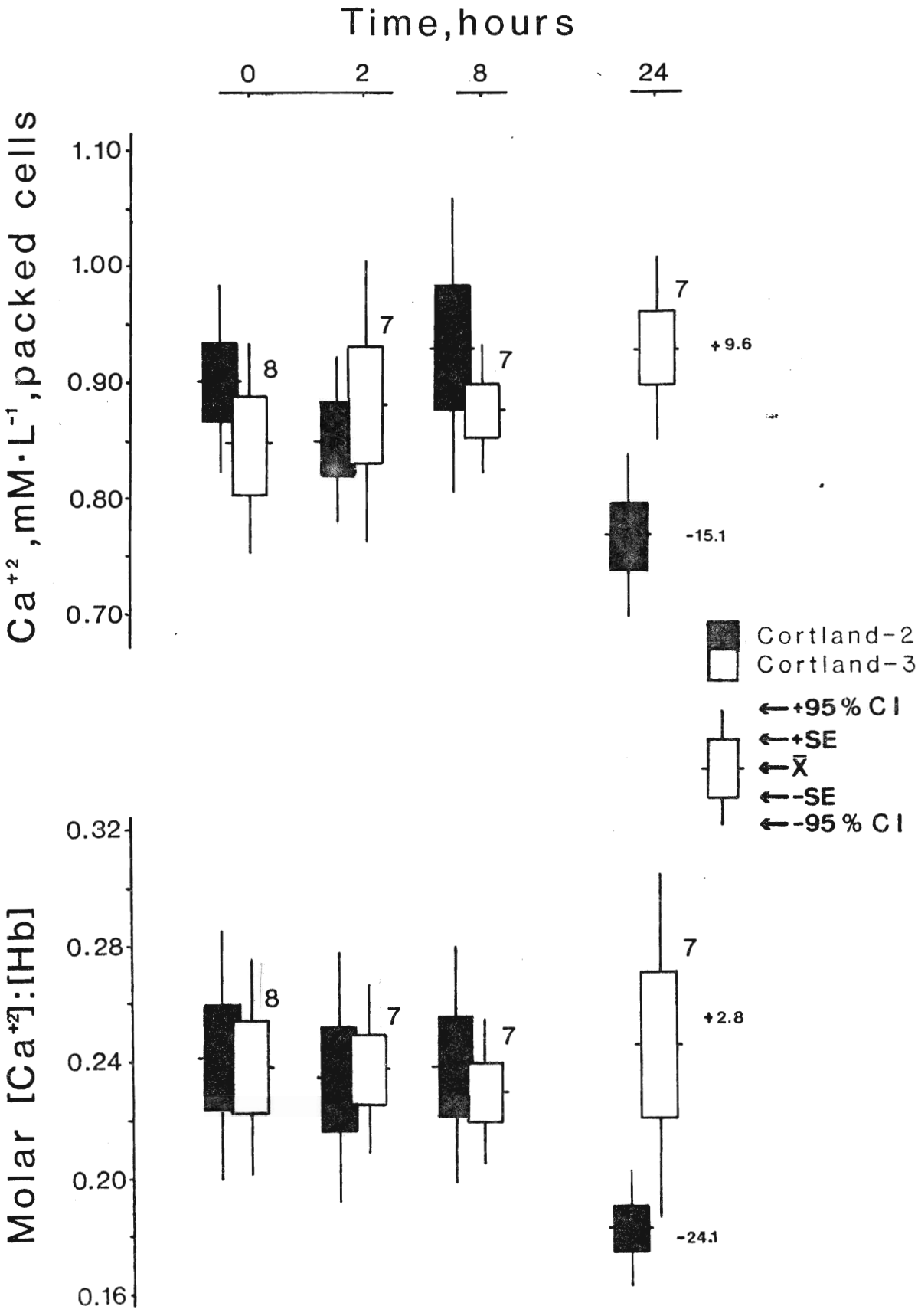
while molar K^{+}:Hb ratios increased slightly (2.4%) over this same period. Parallel studies using noradrenaline-supplemented media displayed a non-significant decrease in potassium content of 3.2%. [K]:[Hb] ratios increased by 4.8% over original values and were not significant. Neither the zero hour nor twenty-four hour values for hormone-treated and non-treated cultures differed significantly in either their potassium levels or [K]:[Hb] ratios.

Red cell magnesium values increased significantly in Cortland-2 studies ($p < 0.01$) with 24 hour values being 29.9% above original levels (Fig. 4, Appendices I, IIb). No significant difference was found in Cortland-3 incubations although a modest increase of 8.6% was observed. Although [Mg]:[Hb] ratios did not change significantly in either series, flasks without hormone displayed an average Mg^{+2} increase of 17.0% as compared to a 1.8% increase in catecholamine-treated cultures. A significant difference was evidenced between 0 hour values of Cortland-2 and -3 studies in Mg^{+2} concentration ($p < 0.05$) and a near significant difference in [Mg]:[Hb] ratio ($p = 0.0505$). No appreciable differences between 24-hour values of hormone and non-hormone treated cultures were detected.

Significant reductions in calcium ion content were witnessed in Cortland-2 incubated red cells over the 24-hour study period (Fig. 5, Appendices I, IIc), both in calcium concentration and calcium:hemoglobin ratio ($p < 0.05$). The magnitude of decline was -15.1% and -24.1% respectively. Hormone- treated red cells showed no significant changes in Ca^{+2} concentration or in

Figure 4. Variations in magnesium ion levels and molar ion:hemoglobin ratio during a 24 hour incubation period in either Cortland-2 (closed boxes, without hormone) or Cortland-3 (open boxes, with hormone) saline. The number above each box-and-whisker diagram represents the n value for each while that in small print to the right is the percent change in mean 24 hour value from mean 0 hour (original) levels. Incubation flasks were maintained at 14 C $\pm$ 0.1 C.

Figure 5. Variations in calcium ion levels and molar ion:hemoglobin ratio during a 24 hour incubation period in either Cortland-2 (closed boxes, without hormone) or Cortland-3 (open boxes, with hormone) saline. The number above each box-and-whisker diagram represents the n value for each while that in small print to the right is the percent change in mean 24 hour value from mean 0 hour (original) level. Flasks were incubated at 14 C ± 0.1 C.


Time, hours
0
2
8
24
1.10
1.00
0.90
0.80
0.70
Ca^{+2}, mM·L^{-1}, packed cells
8
7
7
7
+9.6
-15.1
Cortland-2
Cortland-3
←+95% CI
←+SE
←$\bar{X}$
←-SE
←-95% CI
0.32
0.28
0.24
0.20
0.16
Molar [Ca^{+2}]:[Hb]
8
7
7
7
+2.8
-24.1

relation to hemoglobin values over the incubation period. A modest increase in intracellular calcium of 9.6% occurred while [Ca]:[Hb] ratio increased by 2.8%. Significant differences between 24-hour values for Cortland-2 and -3 cultures were observed in both concentration ($p < 0.01$) and hemoglobin ratio ($p < 0.05$) instances. No statistical difference between zero hour values was evident.

Significant decreases in chloride ion concentration (Fig. 6, Appendices I, IId; -27.1%; $p < 0.05$) were found in studies using Cortland-2 saline while [Cl]:[Hb] ratios displayed a 32.8% reduction ($p < 0.01$). No significant changes were found in catecholamine-treated cells. Final chloride content averaged 4% less than original values, and [Cl]:[Hb] ratio was reduced by 7.6%. No significant differences were found between culture series in either 0- or 24-hour values of chloride concentration and molar chloride:hemoglobin ratio.

3. Hemoglobin and Water Content

Hemoglobin values increased by 10.9% in Cortland-2 red cell incubations over the 24-hour period of incubation. The Cortland-3 series showed a roughly similar increase of 9.6% over a similar time period (Fig. 7, Appendices I, IIe). No significant changes were found between 0 and 24 hour samples in either case. This was also true of comparisons between 0- and 24-hour values for the two culture series.

A decrease of 7.3% in cell water content was found in studies employing Cortland-2 media, while Cortland-3 cultures displayed reduced water losses of 2.8% (Fig. 7, Appendices I,

Figure 6. Variations in chloride ion levels and molar ion:hemoglobin ratio during a 24 hour incubation period in either Cortland-2 (closed boxes, without hormone) or Cortland-3 (open boxes, with hormone) saline. The number above each box-and-whisker diagram represents the n value for each while that in small print to the right is the percent change in mean 24 hour value from mean 0 hour (original) level. Flasks were incubated at 14 C $\pm$ 0.1 C.

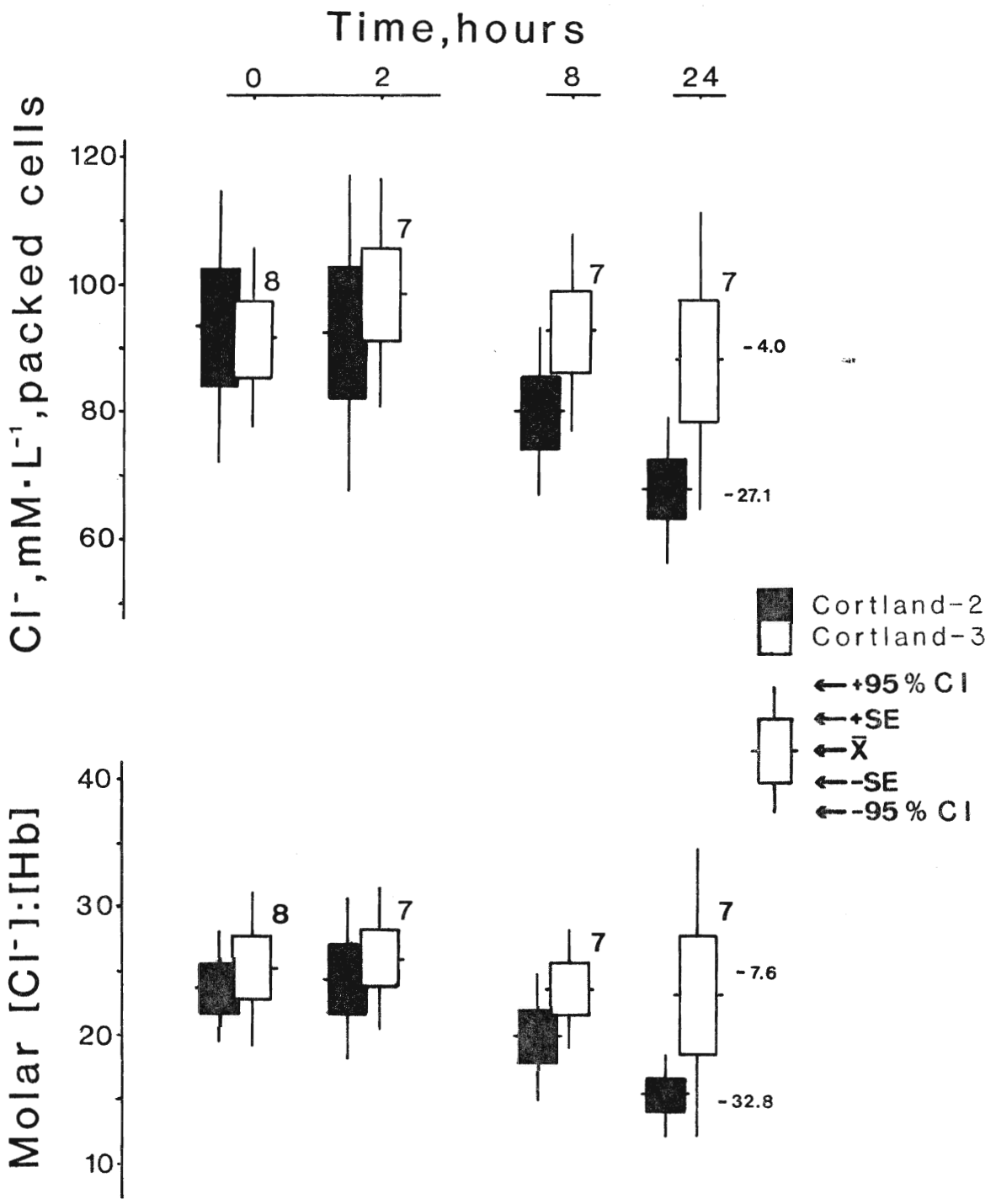
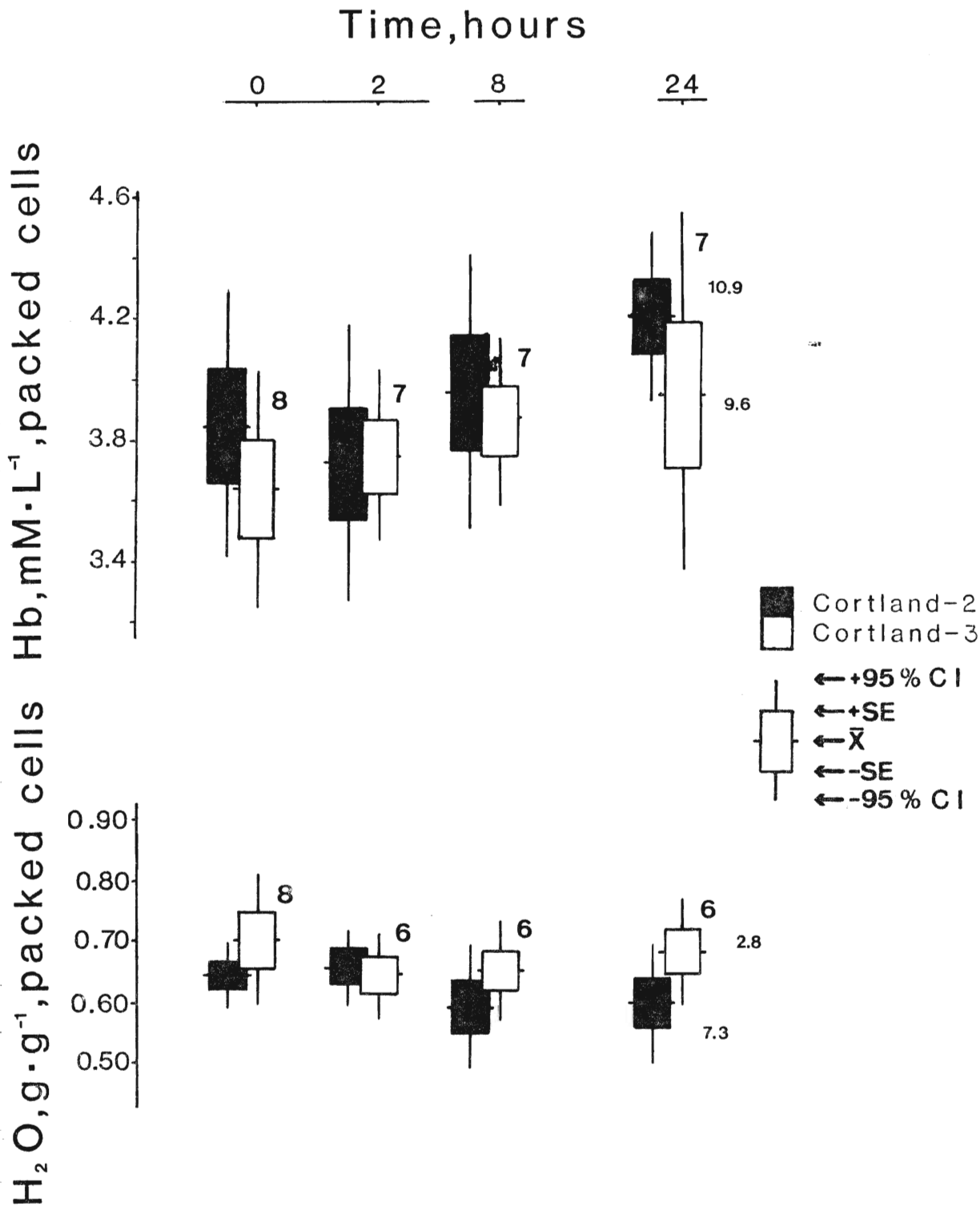

Time, hours
0
2
8
24
120
100
80
60
Cl^-, mM·L^{-1}, packed cells
8
7
7
7
-4.0
-27.1
Cortland-2
Cortland-3
←+95% CI
←+SE
←$\bar{X}$
←-SE
←-95% CI
40
30
20
10
Molar [Cl^-]:[Hb]
8
7
7
7
-7.6
-32.8

Figure 7. Variations in packed cell hemoglobin content and packed cell water content during a 24 hour incubation period in either Cortland-2 (closed boxes, without hormone) or Cortland-3 (with hormone) saline. The number above each box-and-whisker diagram represents the n value for each while that in small print to the right is the percent change in mean 24 hour value from mean 0 hour (original) level. Flasks were incubated at 14 C ± 0.1 C.


Time,hours
0
2
8
24
Hb,mM·L⁻¹,packed cells
4.6
4.2
3.8
3.4
8
7
7
7
10.9
9.6
Cortland-2
Cortland-3
+95 % C I
+SE
X̄
-SE
-95 % C I
H₂O,g·g⁻¹,packed cells
0.90
0.80
0.70
0.60
0.50
8
6
6
6
2.8
7.3

IIf). In neither instance were 24-hour values found to differ significantly from original (0 hour) water contents. No statistical difference was evidenced between original values of Cortland-2 and -3 or in their twenty-four hour values.

The mean increase in hemoglobin content of 10.9% found in Cortland-2 studies was mirrored by a water loss of 7.3% after 24 hours of incubation. This suggested that the increase in hemoglobin content was a consequence of red cell volume reductions. Cortland-3 cultures, however, displayed water losses of 2.8% but a hemoglobin increase of 9.6%. Although it is appreciated that standard errors were relatively large, such findings raise the possibility of catecholamine-induced hemoglobin synthesis. Indeed, further studies in this lab have provided evidence in support of this contention (Houston et al, 1985).

B. Sta-put Characterization and Red Cell Morphology

Following collection of red cells from Sta-put separations, cell counts revealed a near normal distribution of erythrocytes between vials (Fig. 8). Cell recoveries typically averaged 70.8% of the cell load (± 4.9%), a value in accordance with those cited by Miller and Phillips (1973), Pretlow et al (1975), and Lane et al (1982). Erythrocyte concentrations were low in vials containing the heaviest cells i.e. those found in the first layers removed (Table 3, Appendix III). For example, vials A and B accounted for less than 3.0×10^7 cells in total. Cell numbers rose steadily to a maximum in vial F ($\overline{X} = 2.76 \times 10^8$ cells) and

Figure 8. Distribution of recovered erythrocytes following cell separations using the John's/Miller Sta-put™ Apparatus. Vial A represents the first fraction removed from the collecting chamber and vial I the last. Values are the mean ± standard error of estimate. The data are derived from 6-8 cell separations.

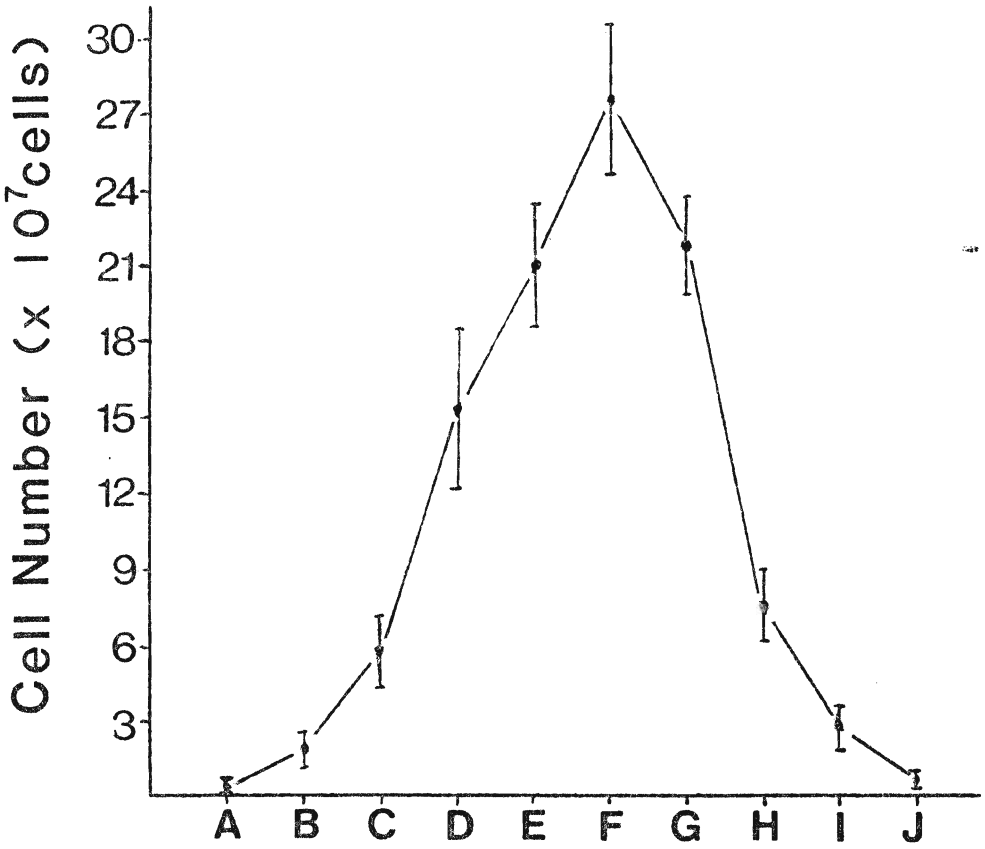

Cell Number (x 10^7cells)
30
27
24
21
18
15
12
9
6
3
A
B
C
D
E
F
G
H
I
J

Table 3. Condescriptive data of cell numbers obtained through cell separations in relation to vial.

Vial	N	Mean ($X\ 10^7$ cells)	SE	95% CI
A	8	0.31	0.16	0.37
B	8	1.85	0.74	1.71
C	8	5.73	1.42	3.27
D	8	15.42	3.21	7.40
E	8	21.05	2.39	5.51
F	8	27.59	2.97	6.85
G	8	21.83	2.02	4.66
H	8	7.75	1.61	3.71
I	8	2.65	0.91	2.10
J	8	0.64	0.32	0.74

then declined sharply (vial I, $\bar{X}$ = 2.71 x 10^7 cells; vial J, $\bar{X}$ = 6.4 x 10^6 cells).

Hemoglobin determinations, and subsequent conversion to mean cell hemoglobin values (Fig. 9, Table 4, Appendix III), revealed the highest cellular hemoglobin levels to be in vials B and C ($\bar{X}$ < 7.0 x 10^{-8} g·cell^{-1}). Most of the cell population, extending over vials D to G, were, however, characterized by lower concentrations, i.e., 5 x 10^{-8} g·cell^{-1}. A sharp decline was evident upon moving to lighter fractions, vial I having a mean cellular hemoglobin content near 1.0 x 10^{-8} g·cell^{-1}. Vials A and J, i.e. the first and last fractions removed from the Sta-put chamber, did not provide enough blood cells to permit accurate analyses.

Microphotographic examination of cellular dimensions (Table 5, Appendix IV) revealed maximal cell lengths to be found in vial B ($\bar{X}$ = 17.47 u) while greatest mean cell width was found in vial E ($\bar{X}$ = 11.30 u). Vial I had the lowest mean value in length ($\bar{X}$ = 11.86 u) and width ($\bar{X}$ = 8.60 u). Overall mean length and mean width for all cells photographed was 15.9 u and 10.6 u respectively. This value is in good agreement with similar morphological data for this species (Iuchi, 1973b; Lane et al, 1982).

Plots of mean cell length and width by vial revealed progressive decreases in size. Vials A to G were relatively uniform in terms of length (Fig. 10), mean values ranging from 17.47-16.51 u. Lighter fractions, H and I, showed a progressive decline from this level having mean cell lengths of 15.00 u and

Figure 9. Mean erythrocytic hemoglobin content as a function of vial fraction following cell separations. Vial A represents the first fraction removed from the collecting chamber and vial I the last. Values are mean ± standard error of estimate. The data are the result of 6-8 cell separations.

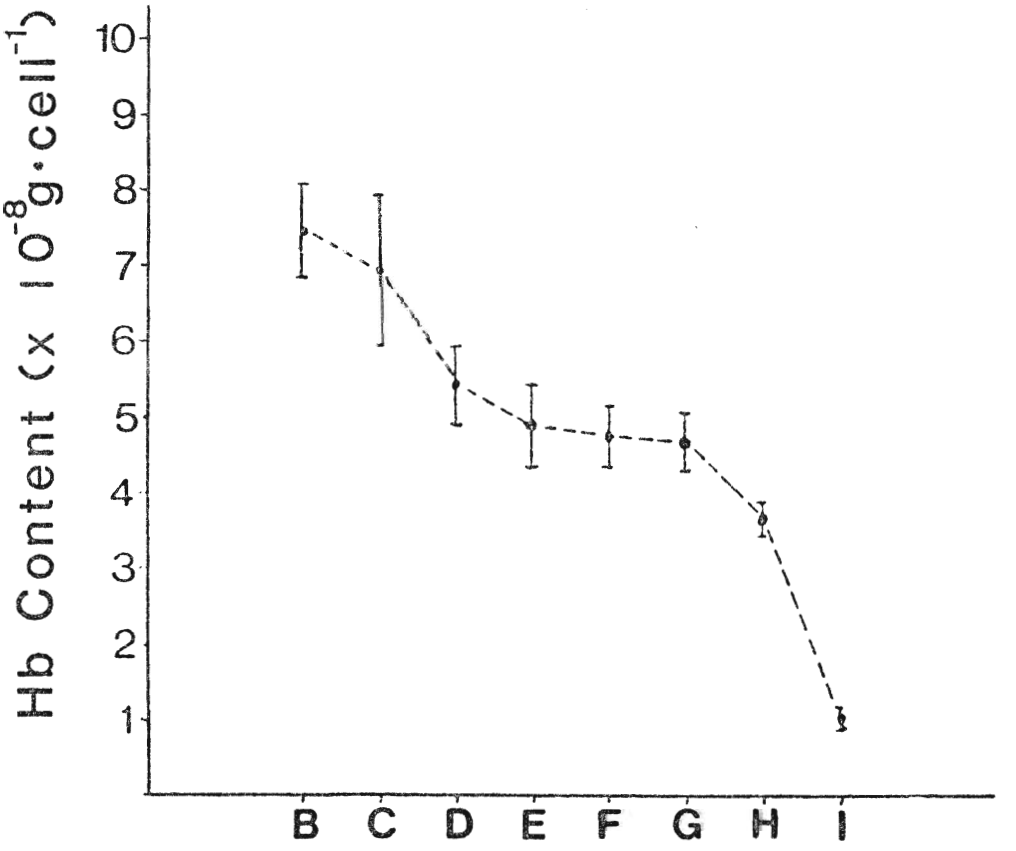

Hb Content (x 10^{-8}g·cell^{-1})
10
9
8
7
6
5
4
3
2
1
B
C
D
E
F
G
H
I

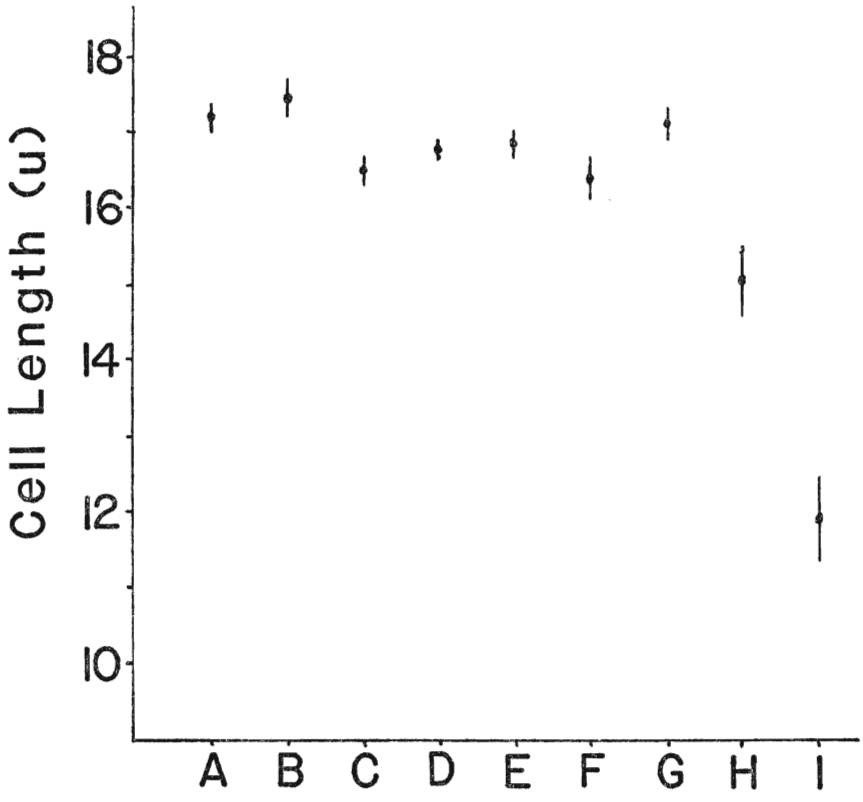
Table 4. Condescriptive data of per erythrocytic hemoglobin content as a function of vial fraction.

Vial	N	Mean ($X\ 10^{-8}$ g·cell^{-1})	SE	95% CI
B	6	7.46	0.50	1.21
C	8	6.98	1.01	2.33
D	8	5.43	0.52	1.19
E	8	4.83	0.54	1.26
F	8	4.75	0.41	0.95
G	8	4.68	0.39	0.90
H	8	3.67	0.21	0.48
I	8	1.01	0.14	0.32

Table 5. Comparison of mean cell lengths and widths on a vial to vial basis. The n values represent the number of photographs examined for each vial and are not meant to impart any information of the proportion of the total cell population each represents. Values listed are mean ± SE.

Vial	N	Mean Cell Length (u)	Mean Cell Width (u)
A	27	17.19 ± 0.20	11.23 ± 0.13
B	35	17.47 ± 0.29	11.16 ± 0.16
C	54	16.51 ± 0.26	10.87 ± 0.22
D	42	16.77 ± 0.14	10.86 ± 0.14
E	31	16.86 ± 0.23	11.30 ± 0.12
F	49	16.37 ± 0.29	11.07 ± 0.17
G	32	17.09 ± 0.22	10.93 ± 0.19
H	48	15.00 ± 0.45	10.41 ± 0.27
I	47	11.86 ± 0.60	8.60 ± 0.35

Figure 10. Mean cell length as a function of vial. All values are mean ± standard error of estimate.


Cell Length (u)
18
16
14
12
10
A
B
C
D
E
F
G
H
I

10.6 μ respectively. A greater degree of variation in cell size was also apparent as characterized by a higher standard error of estimate than in heavier fractions.

Mean cell width (Fig. 11) showed a similar trend. Vials A-G ranged from mean width values of 11.30-10.86 μ. Vials H and I had cells of decreased width averaging 10.41 μ and 8.60 μ. Again standard error of estimates were greater in these fractions, particularly in vial I.

A plot of mean cell length vs mean cell width (Fig. 12) further demonstrates the sudden increase in cell size upon moving to heavier fractions. Mean vial values departed progressively further from the 45-degree line representing equivalence of cell lengths and widths. Thus,increases in length are of proportionally greater magnitude than those of width. Mean cell length:width ratio (Table 6) provides addition evidence for a heightened rate of increase in length. Vial B had the highest ratio value of 1.57 while vial I had a low of 1.35.

Calculation of cell volumes using a prolate spheroid as a mathematical model did not yield realistic data, resulting values being 3-4 times greater than reported trout red cell volumes. Haley and Weiser (1985) reported cellular volumes ranging from 250-550 μ^3 while volumes found in this study often exceeded 1100 μ^3. Relative mean cell volumes (RMCV) for each vial were calculated in an attempt to examine volume variations during maturation. This was done by consideration of the smallest mean estimated volume, found in vial I, as unity and expression of all other vials as a fraction greater than this (Table 7). Greatest

Figure 11. Mean cell width as a function of vial. All values are mean ± standard error of estimate.

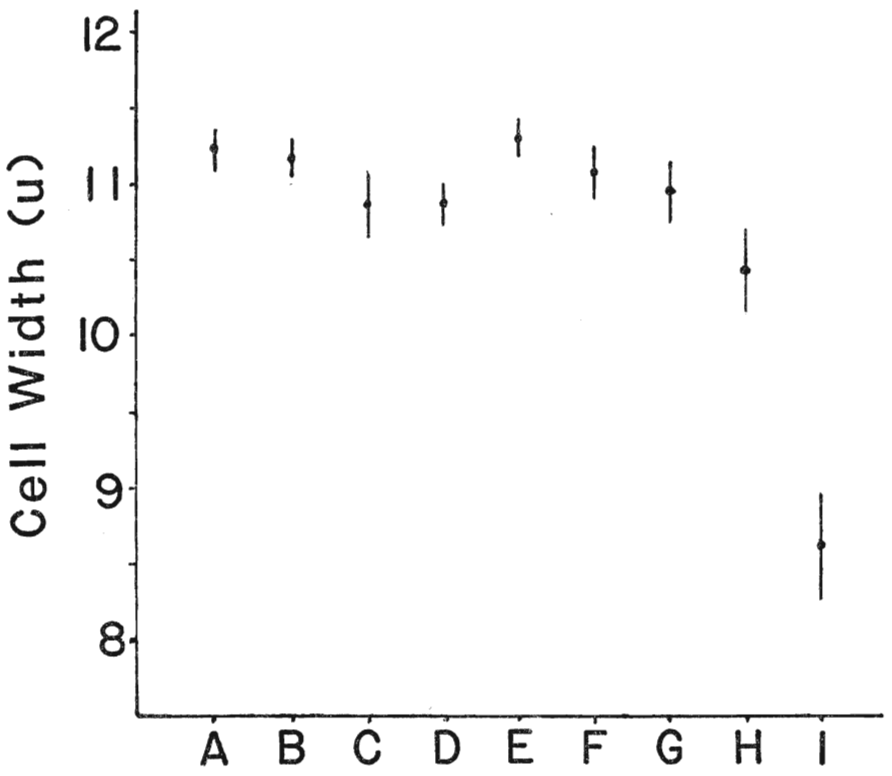

Cell Width (u)
12
11
10
9
8
A
B
C
D
E
F
G
H
I

Figure 12. Mean cell length vs mean cell width. The letters are representative of the vial fractions. Vertical bars are used to demonstrate deviations from the line at which increases in length equal increases in width. Vials A-G have been grouped as they appear to represent a cluster of cells with near equal dimensions.

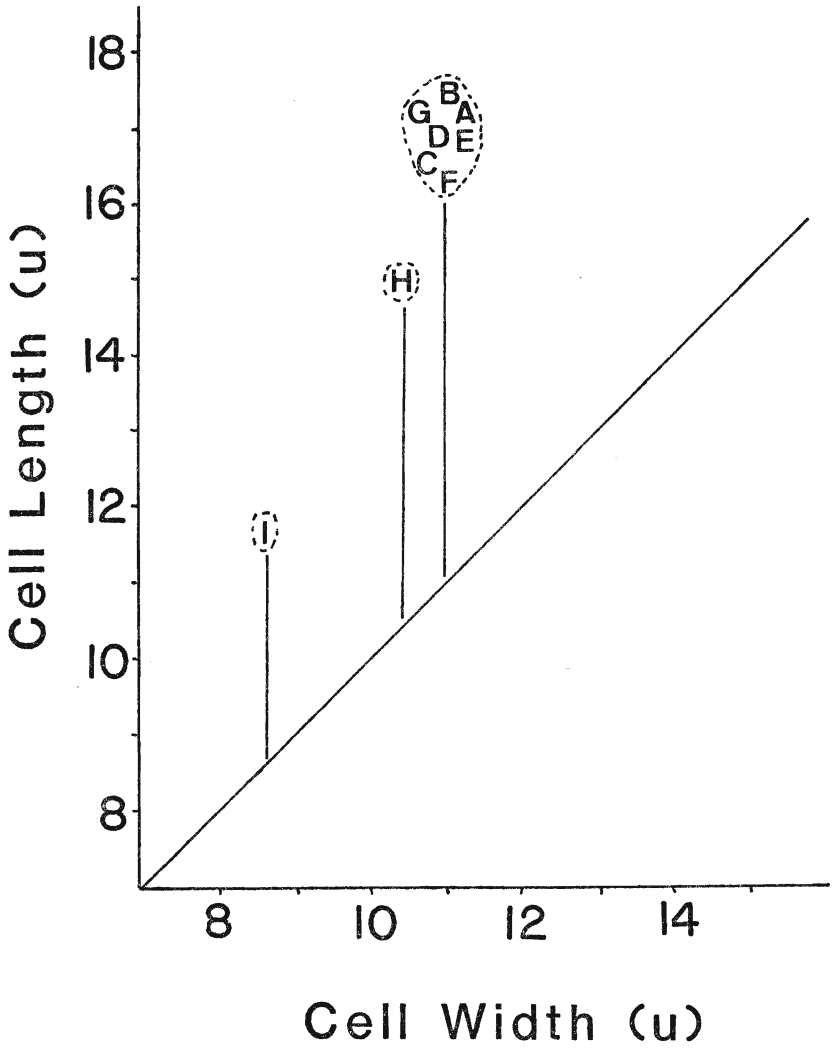

18
16
14
12
10
8
Cell Length (u)
B
G
A
D
E
C
F
H
I
8
10
12
14
Cell Width (u)

Table 6. Comparison of the mean length:width ratio of each cell fraction. The N value represents the number of cell photographs examined in each vial. All values are mean ± SE.

Vial	N	Mean Ratio Length:Width
A	27	1.53 ± 0.02
B	35	1.57 ± 0.02
C	54	1.52 ± 0.02
D	42	1.54 ± 0.01
E	31	1.49 ± 0.02
F	49	1.48 ± 0.02
G	32	1.56 ± 0.02
H	48	1.44 ± 0.02
I	47	1.35 ± 0.03

Table 7. Comparison of the relative mean cell volume of cells (RMCV) found in each vial fraction. The estimated mean cell volume (EMCV) is that determined through use of a prolate spheroid as a mathematical model. RMCV was determined through consideration of the vial possessing the lowest EMCV as unity and expression of all other vials in relation to this. The N value represents the number of cell photographs examined for each vial. EMCV values are mean ± SE.

Vial	N	EMCV (μ^3)	RMCV
A	27	1136.1 ± 33.1	2.48
B	35	1140.6 ± 40.6	2.49
C	54	1022.3 ± 50.3	2.23
D	42	1036.2 ± 32.1	2.26
E	31	1127.9 ± 29.3	2.46
F	49	1051.0 ± 34.3	2.29
G	32	1068.4 ± 44.0	2.33
H	48	853.2 ± 59.8	1.86
I	47	458.7 ± 87.9	1.00

RMCV was found in vial B and was 2.48 times greater than the RMCV of vial I. Vials A-G all were found to have RMCV's in excess of 2.20; vial H had a RMCV of 1.85.

Vial J was not used for examination of morphological changes as it has been shown that the last fraction obtained by this system includes cells which have adhered to the walls of the apparatus. These apparently detach as the last of the fluid leaves the chamber. Consequently, the last vial or vials include a heterogeneous cell population.

A scatterplot of the total cell population (Fig. 13) suggests the possiblility that two subpopulations of cells may exist. The bulk of the cellular population was characterized by a mean length:width ratio of 1.51 and were found primarily in vials A-G (Table 8). Cell lengths ranged from 10.9 to 21.0 μ while cell widths ranged from 14.1 to 8.3 μ. The second subpopulation was found almost exclusively in vials H and I and had a mean length:width ratio of 1.27. The maximal cell length and width in this group was 10.2 μ and 7.9 μ respectively. Student's t-tests revealed these subpopulations to be significantly different in length, width and major:minor axes ratios ($p < 0.01$ in each case).

C. Electrophoresis of hemolysates of separated cells

Visual examination of cellulose acetate strips revealed the presence of a least 11 hemoglobin bands, seven cathodal and four anodal (Fig. 14). All bands, however, were not found in all specimens (see Table 9). Eleven bands were typically seen in samples from vials containing the older, heavier cells

Figure 13. Scatterplot of all photographed cells (n = 377) regardless of vial origin. These are grouped by dashed lines to indicate the possible existence of two cellular populations. Bars represent 95% confidence intervals of the mean length (horizontal) and width (vertical).

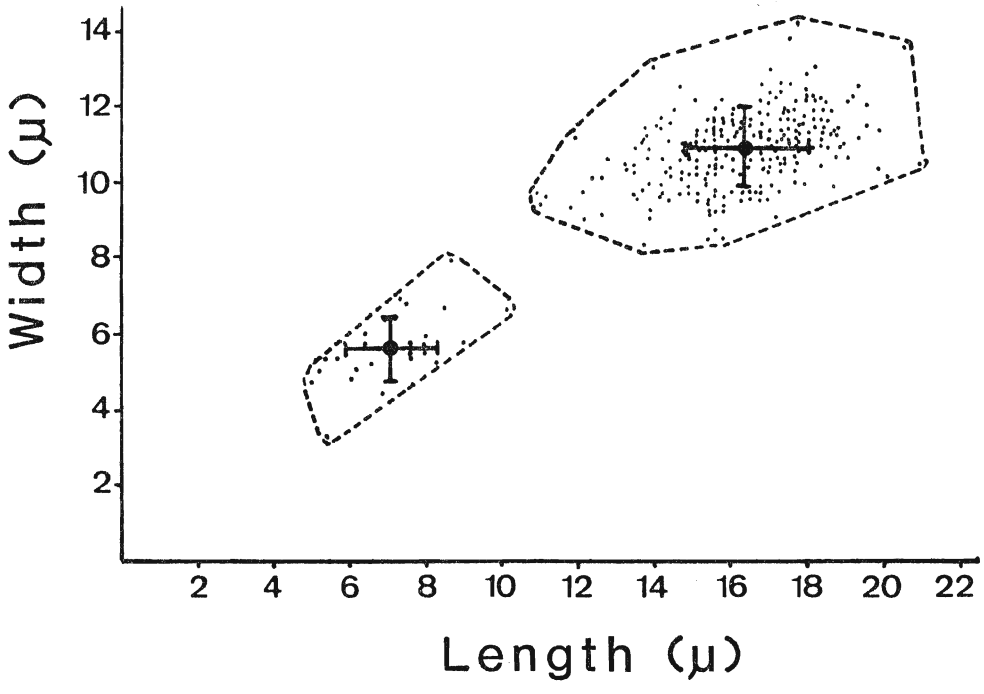
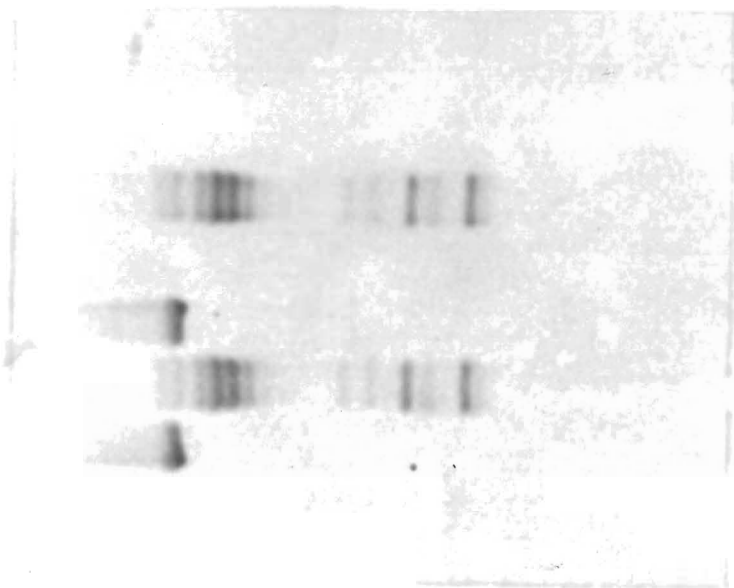
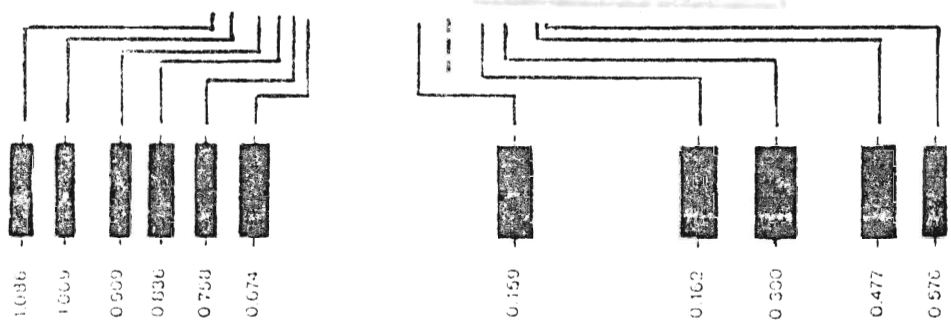

Width (μ)
14
12
10
8
6
4
2
2
4
6
8
10
12
14
16
18
20
22
Length (μ)

Table 8. Condescriptive data on the two subpopulations in existence within the peripheral erythrocytic spectrum.

	Length (u)	Width (u)	L:W Ratio
Population 1			
N	350	350	350
Sum	5795.6	3842.6	528.52
Max	21.0	14.1	2.01
Min	10.9	8.3	1.06
X	16.96	10.98	1.510
SE	0.08	0.05	0.008
95% CI	0.16	0.10	0.016
Population 2			
N	27	27	27
Sum	192.0	151.4	34.39
Max	10.2	7.9	1.63
Min	5.0	3.3	1.00
X	7.11	5.61	1.274
SE	0.25	0.17	0.038
95% CI	0.51	0.35	0.077

Figure 14. Hemoglobin system organization following electrophoresis on cellulose acetate plates. The bar-and-whisker figures represent the mean relative fluidity values (± 95% confidence intervals) determined through comparison with a human hemoglobin standard (Hb A). The tracing at the bottom is representative of that obtained through densitometric analysis. Eleven distinct hemoglobin isomorphs were detected, seven cathodal and four anodal. These were consistent with those found by Tun (1985). The photograph demonstrates the typical hemoglobin system organization displayed by vial C. The dashed line indicates the origin of hemolysate application.




0.159
0.162
0.300
0.477
0.576

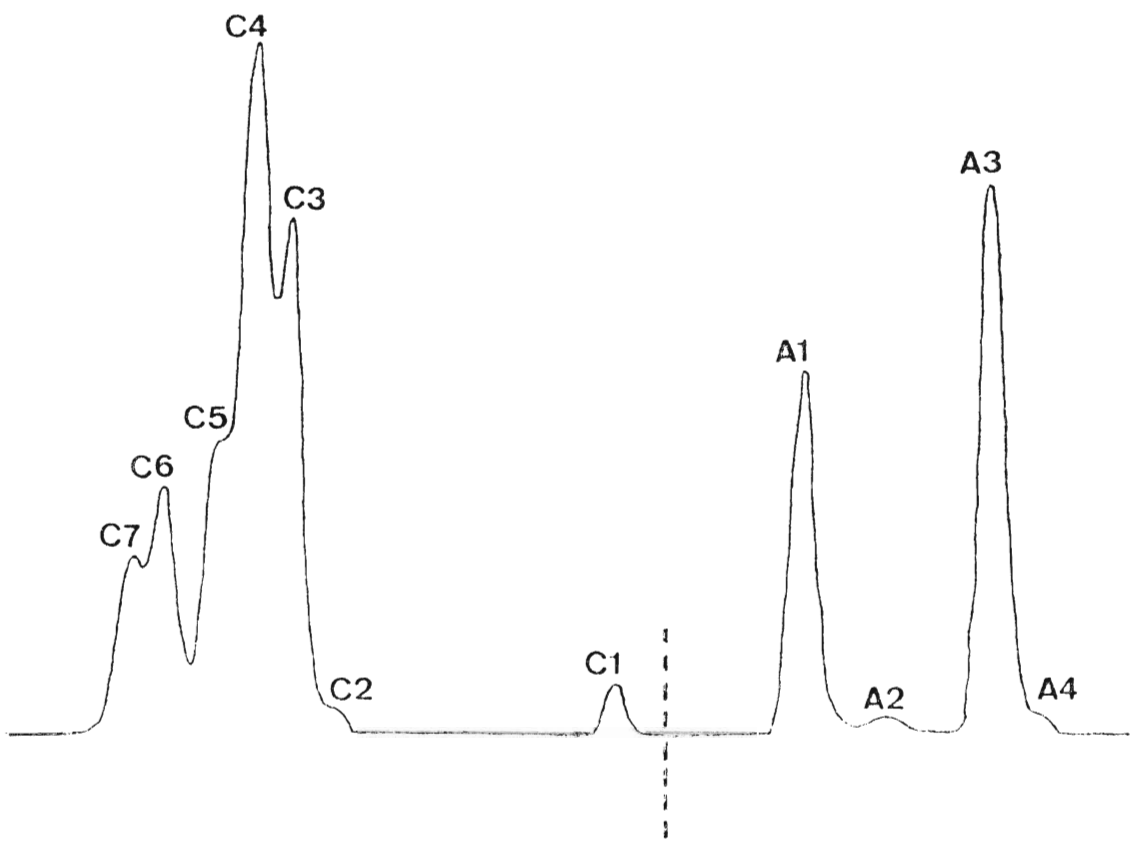

C4
C3
C5
C6
C7
C2
C1
A1
A2
A3
A4

Table 9. Comparison of the percent occurrence of each band on a vial to vial basis. The n values represent the number of electrophoretic runs made on each fraction. A value of 100% indicates that the band under examination was found in all electrophoretic runs of that vial.

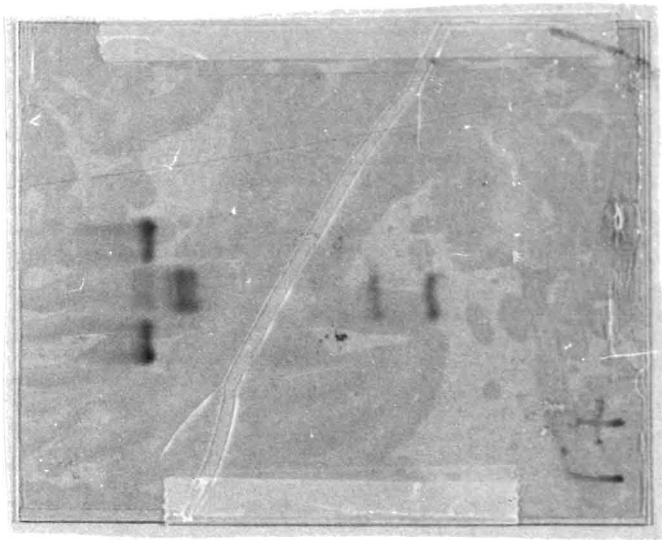
BAND NO.	C	D	E	F	G	H	I
C7	100.0	100.0	100.0	100.0	100.0	100.0	100.0
C6	100.0	100.0	100.0	100.0	100.0	100.0	100.0
C5	100.0	100.0	100.0	100.0	100.0	100.0	100.0
C4	100.0	100.0	100.0	100.0	100.0	100.0	100.0
C3	100.0	100.0	100.0	100.0	100.0	100.0	100.0
C2	100.0	94.4	91.2	75.0	55.6	6.3	0.0
C1	100.0	100.0	100.0	100.0	100.0	100.0	100.0
A1	100.0	100.0	100.0	100.0	100.0	100.0	100.0
A2	100.0	100.0	100.0	100.0	72.2	50.0	5.6
A3	100.0	100.0	100.0	100.0	100.0	100.0	100.0
A4	75.0	77.7	70.1	60.0	38.9	6.3	0.0
n	20	36	34	40	36	32	18

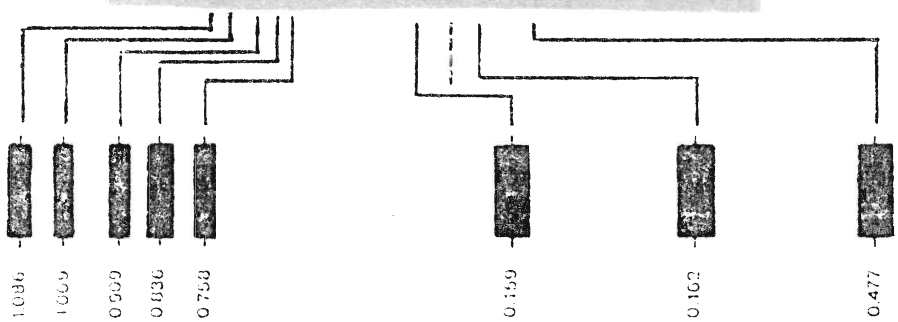
(i.e. vials C to F). Fewer bands were found in lighter, more juvenile fractions. In almost all cases only 8 bands were visualized in cells from vial I (Fig. 15).

One way analysis of variance of relative fluidity values (Rf) demonstrated each band to be distinct ($p < 0.05$). Similarly, no differences were detected in bands between vials, i.e. band C3 for vial C was not significantly different from band C3 of any other vial. Bands C1, C3, C4, C5, C6, C7, A1, and A3 were present at all times in all cell fractions. Bands C2, A2, and A4 were present to varying degrees, dependent upon vial number. In terms of percentage occurrence there was a relatively steady decline in moving from older to younger cell fractions. Thus, Band C2 was found in more than 90% of samples of vials C-E. However its occurrence decreased to a minimum of 6.3% in vial H. Band C2 was never found in cells from vial I. Band A2 was found in all samples from vials C-F. Frequency of occurrence dropped, however, to a low of 5.6% in vial I. Band A4 was not found in all samples. It nevertheless exhibited a similar trend. Vials C-E exhibited occurrence values in excess of 70%. Declining values occurred in moving to younger fractions with band A4 being absent in vial I.

Percent hemoglobin abundancy for each band of each vial fraction, i.e. the percentage contribution of each band to the total hemoglobin complement, are listed in Table 10. Cathodal bands accounted for, on average, 73% of all hemoglobin present while anodal bands comprised an average of 27%. Cathodal band 4 contained the greatest proportion of the total hemoglobin in all

Figure 15. The hemoglobin system organization of hemolyzate obtained from cells of vial I (juvenile). In contrast to vial C, only eight of eleven bands are visualized. The bar-and-whisker figures represent the mean relative fluidity values (± 95% confidence intervals) determined through comparison with a human hemoglobin standard (Hb A). The tracing at the bottom is representative of that obtained through densitometric analysis. The dashed line represents the origin of hemolysate application.




1.036
1.000
0.909
0.836
0.758
0.159
0.162
0.477

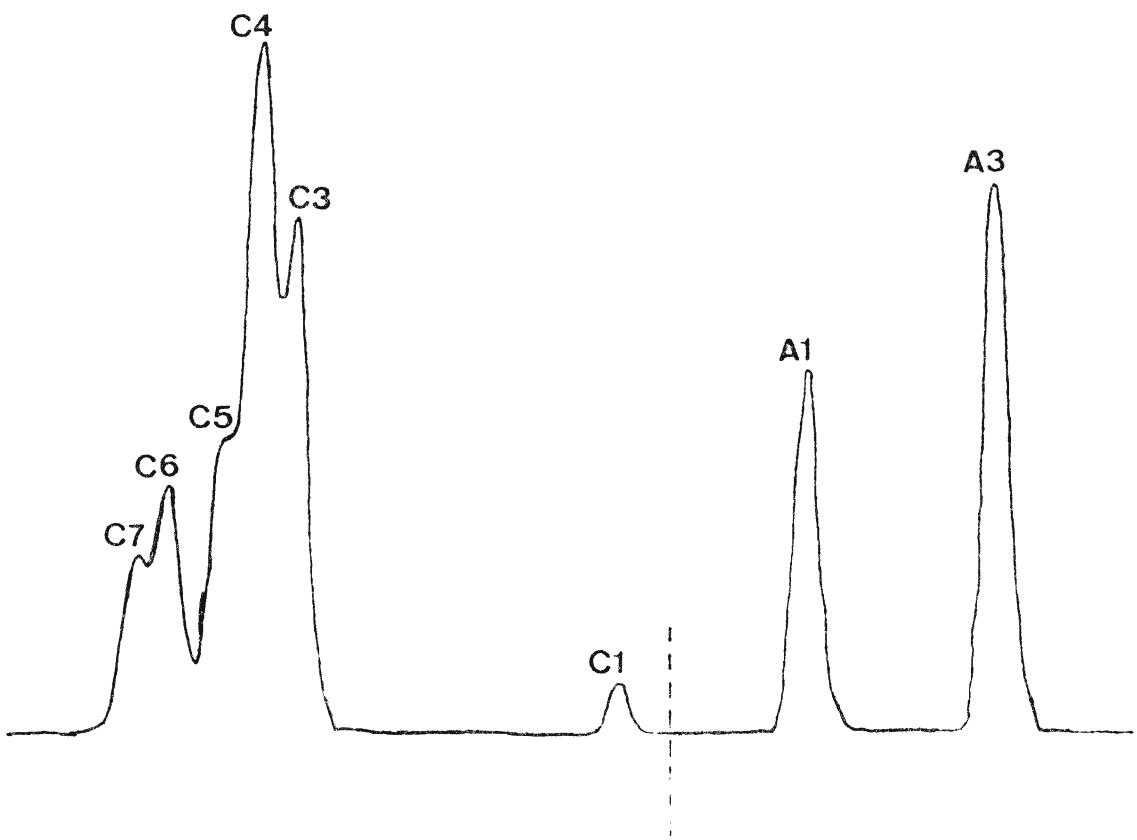

C4
C3
C5
C6
C7
C1
A1
A3

Table **10.** Mean relative hemoglobin abundancy of cathodal and anodal bands as determined from densitometric tracings of cellulose acetate strips. In instances of strips where not all bands were present, a value of 0 was ascribed to the missing band and included in the calculation. Values are percent values, i.e. the total of all band isomorphs in each instance is 100%, and are mean ± SE.

Vial	C7	C6	C5	C4
C	6.86 ± 0.28	7.95 ± 0.28	9.34 ± 0.36	30.29 ± 0.56
D	6.46 ± 0.26	8.00 ± 0.26	9.78 ± 0.33	29.08 ± 0.44
E	6.80 ± 0.21	8.09 ± 0.22	9.92 ± 0.34	28.25 ± 0.39
F	6.46 ± 0.18	7.83 ± 0.21	10.01 ± 0.40	29.04 ± 0.53
G	6.39 ± 0.22	7.37 ± 0.25	11.04 ± 0.39	29.53 ± 0.44
H	6.39 ± 0.20	7.65 ± 0.27	10.33 ± 0.39	29.84 ± 0.49
I	7.10 ± 0.16	8.11 ± 0.20	10.06 ± 0.54	29.31 ± 0.56

	C3	C2	C1	A1
C	18.34 ± 0.19	0.49 ± 0.15	1.01 ± 0.06	10.45 ± 0.35
D	17.71 ± 0.34	0.27 ± 0.07	1.14 ± 0.09	11.55 ± 0.28
E	17.40 ± 0.33	0.37 ± 0.06	1.26 ± 0.11	11.71 ± 0.29
F	17.22 ± 0.31	0.35 ± 0.05	1.35 ± 0.11	11.70 ± 0.27
G	16.91 ± 0.50	0.16 ± 0.05	1.20 ± 0.08	11.38 ± 0.29
H	17.91 ± 0.31	0.02 ± 0.01	0.89 ± 0.07	11.34 ± 0.30
I	18.43 ± 0.22	0.00	1.05 ± 0.08	10.79 ± 0.36

	A2	A3	A4
C	0.45 ± 0.09	14.59 ± 0.37	0.17 ± 0.05
D	0.37 ± 0.07	15.53 ± 0.45	0.17 ± 0.03
E	0.59 ± 0.06	15.42 ± 0.33	0.18 ± 0.30
F	0.53 ± 0.07	15.42 ± 0.26	0.15 ± 0.03
G	0.28 ± 0.06	15.31 ± 0.31	0.06 ± 0.02
H	0.16 ± 0.05	15.49 ± 0.48	0.01 ± 0.01
I	0.02 ± 0.02	15.14 ± 0.64	0.00

cases with an overall mean average of 29.34%. The remainder, in declining order were C3 (17.71%), A3 (15.27%), A1 (11.28%), C5 (10.07%), C6 (7.86%) C7 (6.64%) and C1 (1.13%). The remaining three bands (A2, C2, A4) were not found in all instances and had overall mean average abundancies of 0.34%, 0.24% and 0.11% respectively. Data was found to be non-normally distributed in some instances (Kolgomorov-Smirnov test, 0.05 level) and this condition was not alleviated through arc-sine transformation. Consequently, a Kruskal-Wallis one-way analysis of ranks was employed. No significant differences in major bands was found between vials, that is, the percentage of the hemoglobin complement of vial C represented by band C1 was not significantly different than that represented by band C1 of any other vial at the 0.05 level of significance.

DISCUSSION

Normalcy of Red Cells During Incubations

Recent studies by Houston et al (1985) have demonstrated that cells incubated in Cortland saline do not remain in a condition which approximates cell normalcy. Marked decreases in NTP, water content and cell volume were evident after only two hours of incubation. Increases in Ca^{+2}, Mg^{+2} and Na^{+} were coupled with K^{+} and Cl^{-} reductions.

Many of these findings are consistant with the sequence of events associated with the "Gardos effect" (Gardos, 1959). With reduction in nucleotide triphosphate level the ATP-driven calcium pump is fuel-starved and becomes ineffective maintenance of cellular calcium levels; net Ca^{+2}-influx therefore occurs (Ferreira and Lew, 1977). Calcium ion buildup interferes with the gating mechanism of K^{+}-specific membrane channels, and rapid K^{+} losses occur. Membrane hyperpolarization heightens Ca^{+2}/K^{+} interaction as Ca^{+2} uptake is now promoted. Continuation of these processes causes inhibition of the ATPase-dependent (Na/K) transport system and exacerbates K^{+} loss and Na^{+} accumulation (Yingst and Hoffman, 1984). Membrane hyperpolarization also prompts passive uptake of Mg^{+2}, an event which, given the involvement of magnesium (and calcium) in the regulation of a number of enzymatic processes, can have far-reaching effects on cellular metabolism.

Addition of pyruvate (Cortland-2) appeared to stabilize NTP levels. Volumetric and ionic composition variations were reduced but they were not eliminated (this study). Significant losses of

chloride and, to a lesser extent, calcium ions were still observed while magnesium ion levels were more than double original values.

Given the known interactions of anions with the hemoglobin molecule, decreases in chloride ion availability would be expected to favour the breaking of salt bridges promoting a shift from the deoxy or "tense" (T)-state to the oxy or "relaxed" (R)-form. In doing so hemoglobin-oxygen affinity would increase, favouring oxygen uptake at the gill but acting against release to tissue.

Increases in magnesium ion content and chloride ion reductions, should prompt a shift to the oxygenated R-configuration. This divalent cation influences hemoglobin-oxygen affinity through complexation with organophosphate modulators (ATP, GTP) found in teleostean red cells. These normally exhibit preferential binding between the NH_2-terminal ends of the β-chains of unbound hemoglobin molecules; new salt bridges result and the molecule is stabilized in the deoxy-state (Weber and Lykkeboe, 1978). Binding of phosphates to animo-terminal ends has been studied extensively in mammalian systems (Benesch and Benesch, 1974; Perutz, 1978). It is generally considered that the same sites are involved in all vertebrate hemoglobins (Hol et al, 1978; Houston, 1980). In mammalian erythrocytes the principle organophospate modulator is 2,3-diphosphoglycerate (2,3-DPG). This is functionally substituted in the trout red cell by ATP and, to a lesser extent, GTP. Given constancy of NTP levels, complexation by magnesium

ions render organophosphates unavailable for allosteric interaction with hemoglobin, increasing Hb-O_2 affinity and shifting the oxygen dissociation curve to the left (Weber, 1982). With the addition of pyruvate to Cortland-2 saline, NTP levels were stabilized.

Although significant decreases in calcium were found, and would be expected to prompt switching to the deoxy or T-configuration, free magnesium levels exceeded free calcium concentrations ($\bar{X}$ 0 hour values: Mg^{+2} = 5.29 mM; Ca^{+2} = 0.90 mM) and calcium reductions were minor compared to magnesium increases (Mg^{+2} = +1.58 mM; Ca^{+2} = -0.14 mM). In addition, calcium ion has a lower affinity for organophosphate modulators than magnesium (Bunn et al, 1971) and, consequently, a lesser effect on hemoglobin-oxygen dissociation curves.

Cellular water content exhibited non-significant decreases, mirrored by a non-significant increase in hemoglobin concentration.

Riddick et al (1971) in their examination of duck erythrocytes found that vitro volume could be stabilized through addition of noradrenaline to the incubating medium. Subsequent studies (Haas et al, 1982) demonstrated that this catecholamine stimulated Cl^- dependent, Na^+-K^+ cotransport.

Rainbow trout red cells exposed to hypoxia exhibit increases in cellular volume (Nikinmaa and Huestis, 1984). The process is mediated adrenergically and the increase in volume leads to reductions in cellular concentrations of hemoglobin, organophosphates and impermeable polyanions. Donnan forces prompt H^+

efflux and pH increases (Nikinmaa, 1983). Correspondingly, hemoglobin-oxygen affinity increases and oxygen uptake is increased. Baroin et al (1984) found that a net uptakee in Na^+ occurs upon catecholamine addition and is followed by osmotically-obligated water, resulting in cell volume increases. Na^+ uptake involves two components; the principal factor is a 1:1 entry of Na^+ in exchange for Cl^-. This differs from the electrically neutral Na^+-K^+-$2Cl^-$ cotransport described by Kregenow (1977). A minor contribution to net Na^+ influx, independent of Cl^-, is attributable to a Na^+-H^+ counter-transport system. Bourne and Cossins (1982) reported washed trout cells to increase their potassium levels by 100-250% upon exposure to physiologically-realistic levels of norepinephrine.

Addition of norepinephrine to Cortland-3 did not produce significant changes in K^+ levels but did lead to stabilization of Cl^-, Ca^{+2} and Mg^{+2} concentrations. Given maintenance of Cl^- levels through influx with Na^+ (and perhaps K^+) it may be that previously observed alterations in Ca^{+2} represented no more than passive movement along a gradient. Increases in magnesium ion were also alleviated. The mechanics of magnesium movement are not well understood and evidence for active transport is not compelling (Dunn, 1974). It appears likely that the observed changes represent redistribution of free and membrane-bound magnesium in response to electrochemical gradients within the aqueous phase of the cell (Houston, 1985).

Cell water content was also stabilized to a greater extent than was observed with Cortland-2 saline. However, a

non-significant increase in hemoglobin concentration, not attributable to water losses persisted. This, and further studies (Houston et al, 1985) lend support to the hypothesis that norepinephrine may function in the stimulation of hemoglobin synthesis. In doing so it may represent another level of regulation by means of which the trout can increase oxygen carrying capacity.

Using catecholamine fluorescence histochemistry Donald (1984) studied adrenergic innervation of structures in the gills of rainbow trout. The gill contains in general two pathways, the arterio-arterial pathway of blood delivery and the arterio-venous pathway. The first involves the afferent branchial artery, afferent filamental arteries and afferent lamellar arterioles which lead to the capillaries of the secondary lamellae where gas exchange occurs. The second consists of the nutritive arterioles of the gill which drain into an extensive venous system in the gill filament through efferent arterioles and efferent arteries within the gill arch and each filament. This system ultimately drains into a large branchial vein of the gill arch. Although not found in secondary lamellar tissues, fluorescent nerve fibres were found in gill filaments surrounding afferent and efferent lamellar arterioles as well as afferent and efferent filamental arteries.

By increasing circulation of catecholamines during periods of environmental stress, most notably hypoxia, three responses are envisioned. The first occurs at the gill lamellae where catecholamines prompt vasodilation of the arterio-arterial

pathway. As flow is a negative 4th power function of vessel diameter, rate of blood movement is sharply reduced. This permits blood moving through the gills to come in contact with oxygenated surfaces for longer periods of time and this increases oxygen acquisition. Secondly the spleen is also innervated with norepinephrine-sensitive nerve fibres. Because of this heightened concentrations of catecholamines will likely promote organ contraction and release of stored erythrocytes into peripheral circulation (Fange and Nilsson, 1985). More hemoglobin is then made available for oxygen uptake at gill surfaces to satisfy increases in oxygen demand.

Finally, the possibility of catecholamine-stimulated hemoglobin synthesis raises yet another level of adrenergic involvement in meeting oxygen requirements. Through increases in per cell hemoglobin content, more hemoglobin can be made available to pick up and transfer oxygen to tissues. This third response would represent a more permanent adaptation to the environment whereas the first two are likely of a more temporary nature and could be invoked to meet the immediate respiratory needs of the organism.

Characterization of the separation procedure

Our studies on the John's/Miller Sta-put apparatus have demonstrated the first cells removed from the chamber to be of greater length, width, hemoglobin content and relative cell volume than those removed in later fractions. It is assumed that the cells represented in each fraction (or at least those of the

first few and the last fractions) are of different ontogenctic stages.

In their review of cellular senescence Clark and Shohet (1985) examined the properties of maturing red cells and the procedures used in their separation. Cell age has long been associated with density, maturing cells becoming progressively heavier through incre4ases in hemoglobin and reductions in water content.

Eadie and Brown (1953) injected labelled iron into the bone marrow of the dog. Immature red cells picked up iron and encorporated it as heme, thus fixing it within the cell until the cell is removed from circulation and destroyed. Density centrifugation of blood samples over a number of weeks followed hemoglobin accumulation and cell maturation. Initially radioactive cells were found in the lighter, low-density portion of the separation column. As the cells aged this band moved progressively down the column. After 240 days the label was no longer detected in peripheral circulation. Presumably at this point the now senescent cess were eliminated.

Although recent doubts have been raised regarding the correlation between red cell age and density (Luthra et al, 1979) it still appears to represent a reasonable basis for the separation of cells into different age groups. The efficiency of the procedure is, however, determined by the effects of serveral factors on its resolving power. In the human, erythrocytes are released to circulation in the mature morphological state and subsequently synthesize hemoglobin while in circulation. The

latter underlies the differences in buoyancy between young and old cells. In the trout another factor - shape - comes into play as cells are released as small,round discs which mature into larger elliptical forms.

The John's/Miller velocity sedimentation system separates cells primarily on the basis of density and secondarily on shape. Mature cells, with full hemoglobin complement, are presumably more dense than pre-synthetic juvenile cells. These would be expected to drop more rapidly through the density gradient and be found in the first fractions removed. Thus by established criteria these cells would be considered to be the oldest. Separation by density differences should be complemented by shape changes to provide a greater degree of resolution. As erythrocytes sink they orient to meet the least resistance. As these cells mature they exhibit proportionally greater increases in length than width. This produces an elliptically shaped cell which has a much more streamlined appearance than the round juvenile cell. Consequently, such cells would be expected to move more rapidly through the medium.

Separations using this method produced fractions in which the highest hemoglobin contents were found in the first fractions removed, and the lowest values in the last fractions (Fig. 9). Vials B and C possessed hemoglobin contents in excess of 7×10^{-8} g·cell^{-1} while vials D-G ranged from 4.8-5.5 x 10^{-8} g·cell^{-1}. Within this grouping a progressive decline from vial D to G was observed. Similarly the initial fractions had the greatest mean cellular length and width. Decreases in these morphometric

characteristics were found in fractions removed later in the series (Fig. 10 and 11). Resolving power is further evidenced by examination of the length-to-width ratio (Table 6). A length:width ratio of approximately 1.5 was found in the early fractions. This declined progressively in later fractions. The distinctiveness of the first few vials (B and C) from those found in the middle (vials D-G) was not apparent in terms of length:width ratio but was found in comparison of cellular hemoglobin content. Taken together these findings clearly demonstrate that the velocity sedimentation system is highly selective in terms of density and only moderately so in regard to cell shape. In addition, exposure to pertubing physical forces are minimized using this type of system since the cells simply fall through the medium at unit gravity. Thus, by comparison with centrifugation procedures, it represents a highly desirable method of separating cells and, furthermore, one characterized by a relatively high degree of resolution.

Hemoglobin isomorph composition and functional consequences

A plot of cell width versus length for all cells of vial origin (Fig. 13) indicated the possibility of two distinct subpopulations. The more abundant of these was composed of cells found primarily in vials A-G; the second was made up almost exclusively of cells from vials H and I. The larger population undoubtedly represents the adult cells which are active in respiratory activities and account for the bulk of oxygen transport.

Two possibilities can be suggested for the origin of the

second smaller subpopulation. These may represent juvenile cells which have yet to mature to adult status. If this is the case, the lack of cells of intermediate dimensions between the two populations indicates that maturation occurs in a very short period of time; perhaps as rapidly as in a few hours. This is not unlikely as cell elongation involves simply activation of cytoskeletal and marginal microtubular band elements and probably does not require extended periods of time.

The second possibility is that these cells represent an entirely separate pool of cells. These cells may be the remnant of a population which were released to circulation when the organism was in a more juvenile state, and could even have arisen from a different erythropoietic site. Such a conclusion would be consistent with earlier studies on this species. Iuchi (1973b), for example, found a group of cells of intermediate size which represented a transition from larval cells to those of adults. He termed these "immature adult erythrocytes", and found them in the peripheral circulation of fish up to 2 years of age. Progression from the larval to adult stages was associated with reductions in the abundance of, and their replacement by, adult cells. The subgroup of cells which was observed in this present study may represent such a transitional state, detected only because of the separation procedure employed. Whether or not these immature adults are released from different erythropoietic sites than adult cells cannot be determined from existing data.

Electrophoretic analysis of red cell hemolysates provided some additional, pertinent information but does not resolve the situtation. Instead of an abrupt change in the hemoglobin patterns of rapidly and less rapidly sedimenting cells, there was a gradual decline in the number of isomorphs found. This involved only 3 of the 11 hemoglobin bands visualized; the other 8 were present in all fractions in all instances. Differences in isomorph pattern would be indicative of different cell types. However, mobility data obtained indicated that isomorphs were identical, i.e. band 1 of vial C is the same as band 1 of vial I. Similarly no significant differences in relative abundancies were observed in the 8 consistently present isoforms. Bands not always present were not tested in this manner as the absence of a band (as in bands 6 and 11 of vial I) is obviously different from its presence in another vial.

It is the belief of the author that this small subpopulation represents juvenile red cells which quickly mature to adult status as needed. Support for this is found upon reexamination of the cell distribution profile (Fig. 8). Trout do not normally proliferate red blood cells rapidly. Instead there is a steady-state in which the removal rate of older, senescent cells is approximately equal to the rate at which younger, immature red cells are released to circulation. If the rates of degradation and release are comparable, the number of senescent cells should approximate the number of juvenile cells. In addition, given the long life span of trout erythrocytes, both groups should consist of relatively small numbers of cells.

Our observations provide confirmation of both predictions. The first fractions removed from the separation chamber, A and B, contained < 1% of the cell population. These fractions were also characterized by having, as previously mentioned, the highest hemoglobin content per cell (Fig. 9), a condition expected in senescent and near senescent cells. The last fraction, vial I, also consisted of < 1% of the peripheral cell population, and possessed the least amount of hemoglobin per cell. This is a characteristic feature of juvenile forms.

Consideration of erythrocyte volume information (Table 7) provides additon support for this hypothesis. Haley et al (1985) employed an electronic particle counter in tandem with a computer analyzer to examine volume distribution in circulating trout erythrocytes. As was the case in this study, they also found a small population of cells comprising < 1% of the total cell. This group of cells was characterized by relative mean cellular volumes similar to those found in this study. The trout used by Haley and coworkers were fully adult (> 2 years of age) while those of Iuchi (1973b) were 2 years old or less. It seems unlikely that a remnant population of cells would remain in circulation for such an extensive period of time, or that a source of larval erythrocytes would remain active over this period.

If the foregoing hypothesis is valid, rainbow trout red cells are released to circulation as small spherical discs which quickly increase in length and width during maturation. Increases in hemoglobin content also occurs in circulation.

As adult cells contain nearly five times the amount of hemoglobin per cell as juveniles, hemoglobin synthesis must continue throughout the lifetime of the cell. These increases in hemoglobin content are not confined to selected hemoglobin isomorphs. Instead the entire spectrum of isomorphs is produced and all components are formed.

Although juvenile cells possess fewer hemoglobin components than do adults, these involve only the three most minor components, cathodal band 2 and anodal bands 2 and 4.* These hemoglobins showed a progressive increase in abundancy with increasing cell age and an increase in the likelihood of the bands being found in later cell fractions. Given the small amounts of hemoglobin involved it is difficult to ascribe a major role to them. The remaining 8 bands did not differ significantly in relative abundance. These hemoglobins are probably necessary for the cell to be fully functional in oxygen transport. The three minor bands may only be formed in "fine tuning" the respiratory capacity of the cell. Whether the components arise from degradation and reaggregation of existing components or are the product of de novo synthesis is not known. While juvenile cells with their limited isomorph complement may not be as effective as mature forms during periods of environmental stress, such as hypoxia and temperature increase, they likely are involved in routine oxygenation of blood and delivery to tissues. In not requiring the full development of its hemoglobin complement in order to function in respiratory processes, oxygen carrying capacity is increased although the magnitude of contri-

bution is likely small.

In order to fully determine the origin of this subpopulation experiments allowing observation of the cells over their lifetime in circulation need be devised. With the development of a medium which holds erythrocytes in a state approximating normalcy, and the characterization of a system which separates these cells from the bulk of the cellular population this becomes possible. Using cohort labelling of red cells to follow maturation presents difficulty in determining whether or not the label remains confined to erythropoietic sites. Instead it now becomes possible to remove a quantity of blood from the trout, through catheterization of the animal, and to separate these cells into adults and juveniles. Juvenile cells can then be incubated for a period of time in the presence of a radioactive label such as C^{14}-glycine or an iron isotope and reinjected back into the host organism. Sampling over an extended period of time and subsequent separations permit the progression of this cell species to be observed first hand. If they are indeed juvenile forms and not a remnant of a previous state of growth radioactivity studies should demonstrate cells in earlier fractions to possess increasing levels of the isotope with a concommitant decrease in the level of later, lighter fractions.

CONCLUSIONS

(1) Red cell incubation studies demonstrated an involvement of norepinephrine in regulation of intracellular concentrations of Ca^{+2}, Mg^{+2} and Cl^{-}. The effects appear complex if poorly understood.

(2) In addition to effects related to the maintenance of cell volume, norepinephrine addition appeared to stimulate hemoglobin synthesis.

(3) Use of the John's/Miller Sta-put sedimentation system separating at unit gravity provided a relatively high level of cell resolution in terms of density and shape.

(4) Adult cells, collected in the first fractions, were characterized by greater amounts of hemoglobin and larger values of length, width, volume and major:minor axis ratio. Juvenile red cells possessed less hemoglobin per cell and were of smaller size, volume and major:minor axis ratio.

(5) The number of juvenile red cells approximately equalled that of senescent cells, a situation compatible with a steady-state in which rate of cell replacement equals rate of cell loss.

(6) Electorphoresis of juvenile and adult cell fractions revealed progressive increase in the number of hemoglobin isomorphs with cell maturation. Juveniles were found to have only eight of eleven bands distinguished in adult cell hemolysates. The number of components increased as cells assumed a more adult morphology.

(7) No significant differences in the relative abundance of major hemoglobin isomorphs, i.e. those present in all cell hemolysate fractions in all instances, were detected.

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APPENDICES

Appendix Ia. Comparison of potassium ion levels of red cells maintained in Cortland-2 and -3 saline over a 24-hour incubation period.

Cortland-2

	0 hour		2 hour		8 hour		24 hour	
	$mmol \cdot L^{-1}$	[K]:[Hb]	$mmol \cdot L^{-1}$	[K]:[Hb]	$mmol \cdot L^{-1}$	[K]:[Hb]	$mmol \cdot L^{-1}$	[K]:[Hb]
RBT-01	89.65	22.92	91.78	20.83	100.78	28.10	110.33	28.63
RBT-03	113.37	29.91	95.76	24.63	99.11	23.36	90.38	20.20
RBT-05	66.25	16.16	---	---	92.05	25.81	94.71	23.36
RBT-07	106.09	25.27	110.48	27.84	115.42	32.70	---	---
RBT-09	88.35	25.57	97.87	23.76	105.35	23.04	112.22	27.65
RBT-11	92.54	25.47	90.76	27.61	104.25	22.56	119.97	26.45
RBT-13	89.64	31.26	97.97	32.00	---	---	96.68	25.09
RBT-15	95.92	20.24	105.52	32.44	108.37	30.70	115.02	24.94
n	8	8	7	7	7	7	7	7
Sum	741.81	196.80	690.14	189.11	725.33	186.27	739.31	176.32
X	92.73	24.60	98.59	27.02	103.62	26.61	105.62	25.19
SE	4.92	1.73	2.70	1.62	2.79	1.51	4.34	1.07
95% CI	11.63	4.09	6.61	3.96	6.83	3.71	10.63	2.61

Appendix Ia continued...

Cortland-3 (hormone-supplemented)

	0 hour		2 hour		8 hour		24 hour	
	$mmol \cdot L^{-1}$	[K]:[Hb]	$mmol \cdot L^{-1}$	[K]:[Hb]	$mmol \cdot L^{-1}$	[K]:[Hb]	$mmol \cdot L^{-1}$	[K]:[Hb]
RBT-02	97.26	29.23	101.50	28.89	92.56	25.09	100.65	37.29
RBT-04	107.24	34.69	111.25	31.90	99.24	28.72	103.62	24.17
RBT-06	88.81	22.68	---	---	96.30	24.42	93.65	20.26
RBT-08	97.69	26.39	94.87	26.94	102.59	28.83	---	---
RBT-10	108.14	37.09	104.48	28.32	112.44	27.87	108.14	28.41
RBT-12	100.03	28.43	92.69	26.04	95.22	21.70	99.53	27.19
RBT-14	94.62	22.53	89.78	20.91	---	---	93.25	20.58
RBT-16	98.12	23.11	106.88	26.16	111.13	28.57	116.44	29.29
n	8	8	7	7	7	7	7	7
Sum	791.91	224.24	701.01	189.16	709.49	185.20	715.28	187.19
$\bar{X}$	98.99	28.03	100.14	27.02	101.36	26.46	102.18	26.74
SE	2.24	1.95	3.00	1.27	1.08	1.04	3.10	2.22
95% CI	5.30	4.62	7.35	3.11	2.63	2.56	7.58	5.44

Appendix Ib. Comparison of magnesium ion levels of red cells maintained in Cortland-2 and -3 saline over a 24 hour incubation period

Cortland-2

	0 hour		2 hour		8 hour		24 hour	
	$mmol \cdot L^{-1}$	[Mg]:[Hb]	$mmol \cdot L^{-1}$	[Mg]:[Hb]	$mmol \cdot L^{-1}$	[Mg]:[Hb]	$mmol \cdot L^{-1}$	[Mg]:[Hb]
RBT-01	6.38	1.631	7.07	1.605	7.49	2.089	7.92	2.056
RBT-03	5.45	1.438	5.04	1.296	6.60	1.556	6.69	1.495
RBT-05	4.04	0.985	---	---	4.62	1.296	5.10	1.258
RBT-07	5.35	1.274	4.44	1.119	5.17	1.465	---	---
RBT-09	4.39	1.271	3.55	0.862	7.10	1.553	6.90	1.700
RBT-11	6.02	1.657	6.31	1.920	6.67	1.443	7.32	1.613
RBT-13	5.16	1.799	7.81	2.551	---	---	6.99	1.814
RBT-15	5.52	1.156	5.97	1.835	6.61	1.873	7.14	1.548
n	8	8	7	7	7	7	7	7
Sum	42.31	11.220	40.18	11.118	44.26	11.275	48.06	11.484
$\bar{X}$	5.29	1.403	5.74	1.598	6.32	1.611	6.87	1.641
SE	0.27	0.098	0.57	0.214	0.39	0.104	0.33	0.096
95% CI	0.65	0.233	1.38	0.524	0.96	0.254	0.81	0.234

Appendix Ib continued...

Cortland-3 (hormone-supplemented)

	0 hour		2 hour		8 hour		24 hour	
	$mmol \cdot L^{-1}$	[Mg]:[Hb]	$mmol \cdot L^{-1}$	[Mg]:[Hb]	$mmol \cdot L^{-1}$	[Mg]:[Hb]	$mmol \cdot L^{-1}$	[Mg]:[Hb]
RBT-02	6.01	1.812	6.23	1.773	6.48	1.757	7.01	2.597
RBT-04	5.23	1.692	5.35	1.534	5.10	1.476	5.32	1.241
RBT-06	6.50	1.660	---	---	7.27	1.843	7.05	1.525
RBT-08	5.89	1.591	5.39	1.530	5.98	1.681	---	---
RBT-10	6.22	2.133	6.38	1.729	7.53	1.866	6.97	1.831
RBT-12	5.55	1.577	5.80	1.629	5.55	1.265	5.63	1.538
RBT-14	6.01	1.431	5.76	1.342	---	---	6.55	1.445
RBT-16	6.20	1.460	5.89	1.442	6.64	1.707	6.68	1.680
n	8	8	7	7	7	7	7	7
Sum	47.61	13.356	40.80	10.979	44.55	11.595	45.21	11.857
$\bar{X}$	5.95	1.670	5.83	1.568	6.36	1.656	6.46	1.694
SE	0.14	0.079	0.14	0.058	0.33	0.081	0.27	0.166
95% CI	0.33	0.188	0.38	0.141	0.81	0.199	0.65	0.406

Appendix Ic. Comparison of calcium ion levels of red cells maintained in Cortland-2 and -3 saline over a 24-hour incubation period.

Cortland-2

	0 hour		2 hour		8 hour		24 hour	
	$mmol \cdot L^{-1}$	[Ca]:[Hb]	$mmol \cdot L^{-1}$	[Ca]:[Hb]	$mmol \cdot L^{-1}$	[Ca]:[Hb]	$mmol \cdot L^{-1}$	[Ca]:[Hb]
RBT-01	0.758	0.194	0.748	0.170	0.839	0.234	0.636	0.165
RBT-03	0.893	0.236	0.873	0.225	0.907	0.214	0.730	0.163
RBT-05	0.943	0.230	---	---	1.142	0.320	0.804	0.198
RBT-07	0.886	0.211	0.788	0.199	0.961	0.272	---	---
RBT-09	1.083	0.313	0.914	0.222	0.960	0.210	0.839	0.207
RBT-11	0.963	0.265	0.913	0.278	1.001	0.217	0.849	0.187
RBT-13	0.877	0.306	0.932	0.304	---	---	0.787	0.204
RBT-15	0.814	0.172	0.768	0.236	0.695	0.197	0.716	0.155
n	8	8	7	7	7	7	7	7
Sum	7.217	1.927	5.936	1.634	6.505	1.664	5.361	1.279
$\bar{X}$	0.902	0.241	0.848	0.233	0.929	0.238	0.766	0.183
SE	0.035	0.018	0.029	0.017	0.053	0.016	0.029	0.008
95% CI	0.082	0.043	0.072	0.042	0.128	0.041	0.071	0.020

Appendix Ic continued...

Cortland-3 (hormone-supplemented)

	0 hour		2 hour		8 hour		24 hour	
	mmol·L^{-1}	[Ca]:[Hb]	mmol·L^{-1}	[Ca]:[Hb]	mmol·L^{-1}	[Ca]:[Hb]	mmol·L^{-1}	[Ca]:[Hb]
RBT-02	0.826	0.249	0.885	0.252	0.928	0.252	0.988	0.366
RBT-04	0.947	0.306	0.878	0.252	0.894	0.259	0.935	0.218
RBT-06	0.642	0.164	---	---	0.770	0.195	0.787	0.170
RBT-08	0.835	0.226	0.863	0.245	0.873	0.245	---	---
RBT-10	0.798	0.274	0.648	0.176	0.828	0.205	0.875	0.230
RBT-12	0.916	0.259	0.843	0.237	0.883	0.201	0.944	0.258
RBT-14	0.818	0.195	0.944	0.220	---	---	0.903	0.199
RBT-16	0.988	0.233	1.101	0.269	0.950	0.244	1.057	0.266
n	8	8	7	7	7	7	7	7
Sum	6.770	1.906	6.162	1.651	6.126	1.601	6.489	1.707
$\bar{X}$	0.846	0.238	0.880	0.236	0.875	0.229	0.927	0.244
SE	0.038	0.016	0.051	0.011	0.023	0.010	0.032	0.024
95% CI	0.089	0.038	0.124	0.028	0.056	0.025	0.079	0.059

Appendix Id. Comparison of chloride ion levels of red cells maintained in Cortland-2 and -3 saline over a 24-hour incubation period.

Cortland-2

	0 hour		2 hour		8 hour		24 hour	
	$mmol \cdot L^{-1}$	[Cl]:[Hb]	$mmol \cdot L^{-1}$	[Cl]:[Hb]	$mmol \cdot L^{-1}$	[Cl]:[Hb]	$mmol \cdot L^{-1}$	[Cl]:[Hb]
RBT-01	99.56	25.45	96.20	21.83	82.83	23.10	78.78	20.45
RBT-03	113.28	29.88	124.57	32.04	96.21	22.68	60.42	13.50
RBT-05	128.53	31.35	---	---	93.60	26.25	69.61	17.17
RBT-07	92.97	22.14	125.12	31.52	93.05	26.36	---	---
RBT-09	80.37	23.26	70.61	17.14	59.94	13.11	48.75	12.01
RBT-11	55.29	15.22	50.14	15.25	68.68	14.86	86.74	19.11
RBT-13	63.78	22.87	94.67	30.92	---	---	71.80	18.63
RBT-15	118.92	25.80	86.67	26.64	67.92	19.24	62.10	13.47
n	8	8	7	7	7	7	7	7
Sum	752.70	194.63	647.98	175.34	562.23	145.60	478.20	114.34
$\bar{X}$	94.09	24.33	92.57	25.05	80.32	20.80	68.31	16.33
SE	9.11	1.77	10.26	2.66	5.57	1.99	4.75	1.25
95% CI	21.93	4.18	25.10	6.51	13.62	4.87	11.61	3.05

Appendix Id continued...

Cortland-3 (hormone-supplemented)

	0 hour		2 hour		8 hour		24 hour	
	$mmol \cdot L^{-1}$	[Cl]:[Hb]	$mmol \cdot L^{-1}$	[Cl]:[Hb]	$mmol \cdot L^{-1}$	[Cl]:[Hb]	$mmol \cdot L^{-1}$	[Cl]:[Hb]
RBT-02	99.98	30.14	113.69	32.36	128.11	34.73	132.40	49.06
RBT-04	109.86	35.54	123.07	35.28	82.13	23.77	86.25	20.12
RBT-06	64.69	16.52	---	---	75.88	19.24	48.31	10.45
RBT-08	87.89	23.74	96.70	27.46	90.32	25.39	---	---
RBT-10	99.50	34.12	85.30	23.12	92.29	22.87	98.87	25.98
RBT-12	70.94	20.16	70.65	19.85	88.43	20.15	77.68	21.22
RBT-14	109.42	26.05	115.40	26.88	---	---	91.66	20.23
RBT-16	92.05	21.68	85.97	21.04	93.44	24.02	81.54	20.51
n	8	8	7	7	7	7	7	7
Sum	734.33	207.95	690.78	185.99	650.60	170.17	616.71	167.57
$\bar{X}$	91.79	25.99	98.68	26.57	92.94	24.31	88.10	23.94
SE	5.90	2.10	7.29	2.17	6.31	1.92	9.55	4.54
95% CI	13.95	5.67	17.83	5.31	15.44	4.70	23.36	11.10

Appendix Ie. Comparison of hemoglobin concentration of red cells maintained in Cortland-2 and -3 saline over a 24 hour incubation period.

Cortland-2

	0 hour $mmol \cdot L^{-1}$	2 hour $mmol \cdot L^{-1}$	8 hour $mmol \cdot L^{-1}$	24 hour $mmol \cdot L^{-1}$
RBT-01	3.912	4.406	3.586	3.853
RBT-03	3.791	3.888	4.242	4.475
RBT-05	4.100	---	3.566	4.054
RBT-07	4.199	3.969	3.530	---
RBT-09	3.455	4.119	4.572	4.058
RBT-11	3.633	3.287	4.622	4.538
RBT-13	2.868	3.062	---	3.853
RBT-15	4.739	3.253	3.530	4.611
n	8	7	7	7
Sum	30.697	25.984	27.648	29.442
$\bar{X}$	3.837	3.712	3.950	4.206
SE	0.196	0.193	0.193	0.124
95% CI	0.463	0.471	0.471	0.302

Appendix Ie continued...

Cortland-3 (hormone-supplemented)

	0 hour $mmol \cdot L^{-1}$	2 hour $mmol \cdot L^{-1}$	8 hour $mmol \cdot L^{-1}$	24 hour $mmol \cdot L^{-1}$
RBT-02	3.317	3.513	3.689	2.699
RBT-04	3.091	3.488	3.455	4.287
RBT-06	3.916	---	3.944	4.623
RBT-08	3.702	3.522	3.558	---
RBT-10	2.916	3.690	4.035	3.806
RBT-12	3.519	3.560	4.388	3.661
RBT-14	4.200	4.293	---	4.532
RBT-16	4.246	4.086	3.890	3.976
n	8	7	7	7
Sum	28.907	26.152	26.959	27.584
$\bar{X}$	3.613	3.736	3.851	3.940
SE	0.174	0.122	0.120	0.248
95% CI	0.412	0.298	0.293	0.605

Appendix If. Comparison of cell water contents of red cells maintained in Cortland-2 or -3 saline over a 24-hour incubation period.

Cortland-2

	0 hour $g \cdot g^{-1}$ pc	2 hour $g \cdot g^{-1}$ pc	8 hour $g \cdot g^{-1}$ pc	24 hour $g \cdot g^{-1}$ pc
RBT-01	0.6353	0.6714	0.4837	0.4672
RBT-03	0.5301	0.5552	0.6850	---
RBT-05	0.7276	---	0.6599	0.5727
RBT-07	0.6472	---	---	0.5233
RBT-09	----	0.6384	0.6676	0.6931
RBT-11	0.6508	0.7246	0.5975	---
RBT-13	0.6654	0.7312	---	0.6207
RBT-15	0.6507	0.6259	0.4632	0.7052
n	7	7	6	6
Sum	4.5071	3.9467	3.5569	3.5822
$\bar{X}$	0.6439	0.6578	0.5928	0.5970
SE	0.0221	0.0271	0.0397	0.0385
95% CI	0.0542	0.0696	0.1021	0.0988

Appendix If continued...

Cortland-3 (hormone-supplemented)

	0 hour $g \cdot g^{-1}$ pc	2 hour $g \cdot g^{-1}$ pc	8 hour $g \cdot g^{-1}$ pc	24 hour $g \cdot g^{-1}$ pc
RBT-02	0.7969	0.7580	0.5606	0.5884
RBT-04	0.7072	0.5665	---	0.7375
RBT-06	0.4750	---	0.6181	---
RBT-08	0.6756	0.6654	0.6236	0.6052
RBT-10	0.8392	0.6171	0.7848	0.7760
RBT-12	0.8493	---	0.6907	---
RBT-14	0.6084	0.6669	---	0.6248
RBT-16	0.6479	0.5812	0.6289	0.7469
n	8	6	6	6
Sum	5.5995	3.8551	3.9068	4.0788
$\bar{X}$	0.6999	0.6425	0.6511	0.6798
SE	0.0450	0.0287	0.0316	0.0337
95% CI	0.1064	0.0737	0.0813	0.0866

Appendix IIa. Student's t-tests of potassium contents of both hormone-treated and untreated red blood cell cultures.

Medium	Time Period	Units	t	p	
Cortland-2	0 hr vs 24 hr	$mmol \cdot L^{-1}$	-1.939	0.0720	NS
		[K]:[Hb]	-0.279	0.6882	NS
Cortland-3	0 hr vs 24 hr	$mmol \cdot L^{-1}$	-0.851	0.4145	NS
		[K]:[Hb]	0.437	0.6509	NS
Cortland-2 vs Cortland-3	0 hr vs 0 hr	$mmol \cdot L^{-1}$	-1.159	0.2653	NS
		[K]:[Hb]	-1.314	0.2082	NS
	24 hr vs 24 hr	$mmol \cdot L^{-1}$	0.644	0.5368	NS
		[K]:[Hb]	-0.630	0.5451	NS

Appendix IIb. Student's t-tests of magnesium content of both hormone-treated and non-treated red blood cell cultures.

Medium	Time Periods	Units	t	p	
Cortland-2	0 hr vs 24 hr	$mmol \cdot L^{-1}$	-3.714	0.0020	**
		[Mg]:[Hb]	-1.726	0.1051	NS
Cortland-3	0 hr vs 24 hr	$mmol \cdot L^{-1}$	-1.749	0.1010	NS
		[Mg]:[Hb]	-0.138	0.6159	NS
Cortland-2 vs Cortland-3	0 hr vs 0 hr	$mmol \cdot L^{-1}$	-2.15	0.0474	*
		[Mg]:[Hb]	-2.116	0.0505	NS
	24 hr vs 24 hr	$mmol \cdot L^{-1}$	0.963	0.3571	NS
		[Mg]:[Hb]	-0.278	0.6881	NS

Appendix IIc. Student's t-tests of calcium contents of both hormone-treated and non-treated red blood cell cultures.

Medium	Time Periods	Units	t	p	
Cortland-2	0 hr vs 24 hr	$mmol \cdot L^{-1}$	2.967	0.0106	*
		[Ca]:[Hb]	2.812	0.0142	*
Cortland-3	0 hr vs 24 hr	$mmol \cdot L^{-1}$	-1.595	0.1319	NS
		[Ca]:[Hb]	-0.201	0.6657	NS
Cortland-2 vs Cortland-3	0 hr vs 0 hr	$mmol \cdot L^{-1}$	1.087	0.2959	NS
		[Ca]:[Hb]	0.110	0.5812	NS
	24 hr vs 24 hr	$mmol \cdot L^{-1}$	-3.724	0.0030	**
		[Ca]:[Hb]	-2.425	0.0306	*

Appendix IId. Student's t-tests of chloride contents of both hormone-treated and non-treated red blood cell cultures.

Medium	Time Periods	Units	t	p	
Cortland-2	0 hr vs 24 hr	$mmol \cdot L^{-1}$	2.366	0.0327	*
		[Cl]:[Hb]	3.589	0.0030	**
Cortland-3	0 hr vs 24 hr	$mmol \cdot L^{-1}$	0.338	0.6840	NS
		[Cl]:[Hb]	0.416	0.6601	NS
Cortland-2 vs Cortland-3	0 hr vs 0 hr	$mmol \cdot L^{-1}$	-0.209	0.6701	NS
		[Cl]:[Hb]	-0.559	0.5862	NS
	24 hr vs 24 hr	$mmol \cdot L^{-1}$	-1.856	0.0856	NS
		[Cl]:[Hb]	-1.616	0.1294	NS

Appendix IIe. Student's t-tests of hemoglobin contents of both hormone-treated and untreated red blood cells cultures.

Medium	Time Periods	Units	t	p	
Cortland-2	0 hr vs 24 hr	$mmol \cdot L^{-1}$	-1.539	0.1451	NS
Cortland-3	0 hr vs 24 hr	$mmol \cdot L^{-1}$	-1.103	0.2904	NS
Cortland-2 vs Cortland-3	0 hr vs 0 hr	$mmol \cdot L^{-1}$	0.854	0.4120	NS
	24 hr vs 24 hr	$mmol \cdot L^{-1}$	0.959	0.3587	NS

Appendix IIf. Student's t-tests of cell water contents in both hormone-treated and untreated red blood cell cultures.

Medium	Time Periods	Units	t	p
Cortland-2	0 hr vs 24 hr	$g \cdot g^{-1}$, pc	1.095	0.2971 NS
Cortland-3	0 hr vs 24 hr	$g \cdot g^{-1}$, pc	0.336	0.6844 NS
Cortland-2 vs Cortland-3	0 hr vs 0 hr	$g \cdot g^{-1}$, pc	-1.067	0.3061 NS
	24 hr vs 24 hr	$g \cdot g^{-1}$, pc	-1.619	0.1340 NS

Appendix III. Cell numbers, hemoglobin content and hemoglobin content per cell following cell separations.

VIAL A

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$g cell^{-1})
01	0.09	---	---
02	1.35	---	---
03	0.07	---	---
04	0.34	---	---
05	0.06	---	---
06	0.53	---	---
07	0.03	---	---
08	0.01	---	---
n	8		
$\bar{X}$	0.31		
SE	0.16		
95% CI	0.37		

VIAL B

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$g cell^{-1})
01	0.58	0.322	5.55
02	5.25	3.877	7.38
03	1.06	0.685	6.44
04	0.82	0.780	9.48
05	2.04	1.794	8.79
06	4.90	3.491	7.12
07	0.08	---	---
08	0.09	---	---
n	8	6	6
$\bar{X}$	1.85	1.825	7.46
SE	0.74	0.623	0.50
95% CI	1.71	1.601	1.21

Appendix III continued...

VIAL C

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$ g·cell^{-1})
01	4.55	3.580	7.87
02	12.80	11.042	8.63
03	6.96	4.650	6.68
04	5.17	3.431	6.64
05	6.91	4.668	6.75
06	8.20	5.145	6.27
07	0.95	0.572	6.04
08	0.31	---	---
n	8	7	7
$\bar{X}$	5.73	4.727	6.98
SE	1.42	1.198	0.35
95% CI	3.27	2.933	0.85

VIAL D

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$ g·cell^{-1})
01	17.00	9.787	5.76
02	16.05	8.501	5.30
03	31.80	22.326	7.02
04	17.60	11.569	6.57
05	19.70	6.860	3.48
06	14.00	10.061	7.19
07	4.63	1.766	3.82
08	2.61	1.114	4.26
n	8	8	8
$\bar{X}$	15.42	8.998	5.43
SE	3.21	2.335	0.52
95% CI	7.40	5.522	1.19

Appendix III continued...

VIAL E

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$ g·cell^{-1})
01	24.20	8.839	3.65
02	20.00	11.868	5.93
03	26.88	11.853	4.41
04	24.60	13.716	5.58
05	35.70	18.347	5.14
06	20.00	15.079	7.54
07	10.96	3.201	2.93
08	11.92	4.529	3.80
n	8	8	8
$\bar{X}$	21.05	10.929	4.83
SE	2.39	1.826	0.54
95% CI	5.51	4.319	1.26

VIAL F

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$ g·cell^{-1})
01	22.50	13.035	5.79
02	25.60	10.919	4.27
03	30.60	14.195	4.64
04	30.20	14.683	4.86
05	35.70	18.347	5.14
06	41.00	27.474	6.70
07	16.55	5.309	3.21
08	18.60	6.298	3.39
n	8	8	8
$\bar{X}$	27.59	13.783	4.75
SE	2.97	2.485	0.41
95% CI	6.85	5.878	0.95

Appendix III continued...

VIAL G

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$ g·cell^{-1})
01	28.88	13.211	4.57
02	16.53	8.583	5.19
03	29.87	15.244	5.10
04	13.80	7.753	5.62
05	17.90	8.730	4.88
06	24.00	14.424	6.01
07	21.84	6.286	2.88
08	21.85	7.037	3.22
n	8	8	8
$\bar{X}$	21.83	10.159	4.68
SE	2.02	1.257	0.39
95%CI	4.66	2.972	0.90

VIAL H

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$ g·cell^{-1})
01	6.24	2.003	3.21
02	5.43	1.844	3.40
03	3.62	1.536	4.24
04	6.01	2.584	4.30
05	4.70	1.489	3.17
06	6.10	2.760	4.52
07	15.95	5.381	3.73
08	13.96	4.399	3.15
n	8	8	8
$\bar{X}$	7.75	2.750	3.67
SE	1.61	0.502	0.21
95% CI	3.71	1.188	0.48

Appendix III continued...

VIAL I

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$ g·cell^{-1})
01	1.86	0.174	0.94
02	2.87	0.285	0.99
03	1.10	0.125	1.13
04	1.30	0.113	0.87
05	1.66	0.151	0.91
06	2.70	0.249	0.92
07	8.75	0.389	0.45
08	1.43	0.263	1.84
n	8	8	8
$\bar{X}$	2.65	0.219	1.01
SE	0.91	0.033	0.14
95% CI	2.10	0.080	0.32

VIAL J

Run no.	Number of cells ($\times 10^7$ cells)	Amount Hb (g)	Hb/cell ($\times 10^{-8}$ g·cell^{-1})
01	0.14	---	---
02	0.16	---	---
03	0.28	---	---
04	0.44	---	---
05	0.74	---	---
06	0.52	---	---
07	2.80	---	---
08	0.04	---	---
n	8		
$\bar{X}$	0.64		
SE	0.32		
95% CI	0.74		

Appendix IV. Length, width and length:width ratios of cells collected in vial fractions following cell separations. Cell measurements were determined from microphotographs.

VIAL A

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	16.8	10.8	1.56
02	16.1	11.9	1.35
03	15.1	11.2	1.35
04	15.6	10.3	1.51
05	17.0	11.3	1.50
06	16.8	9.9	1.69
07	16.8	10.9	1.54
08	18.4	10.9	1.70
09	18.2	11.8	1.54
10	18.4	10.4	1.77
11	16.1	10.6	1.52
12	17.5	10.9	1.61
13	16.3	11.1	1.47
14	16.3	10.9	1.50
15	18.2	11.4	1.60
16	16.8	11.2	1.50
17	18.6	11.6	1.60
18	17.4	11.8	1.47
19	16.6	11.4	1.46
20	18.4	11.0	1.67
21	16.3	11.8	1.38
22	17.4	12.2	1.43
23	19.2	11.2	1.71
24	16.8	12.4	1.35
25	18.0	12.6	1.43
26	17.4	10.8	1.61
27	17.6	10.8	1.63

VIAL B

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	16.5	11.2	1.47
02	17.2	10.6	1.62
03	14.9	11.0	1.35
04	14.2	11.5	1.23
05	16.3	11.0	1.48
06	15.6	10.8	1.44
07	15.4	9.9	1.56
08	15.4	10.1	1.52
09	16.3	10.1	1.61
10	13.5	9.6	1.41
11	16.1	11.3	1.42
12	17.0	9.9	1.72
13	17.3	10.4	1.66
14	18.7	11.3	1.65
15	21.0	11.6	1.81
16	16.1	11.3	1.42
17	18.4	12.2	1.51
18	17.3	12.0	1.44
19	18.2	12.0	1.52
20	18.6	11.2	1.66
21	19.6	10.4	1.88
22	18.8	11.3	1.66
23	17.8	14.1	1.26
24	18.0	12.4	1.45
25	19.6	11.4	1.72
26	17.2	12.2	1.41
27	17.8	11.4	1.56
28	17.4	11.8	1.47
29	18.6	10.8	1.72
30	18.8	10.6	1.77
31	17.4	11.2	1.55
32	19.0	11.0	1.73
33	17.6	12.6	1.40
34	19.9	10.3	1.93
35	20.1	10.1	1.99

Appendix IV continued...

VIAL C

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	17.9	10.8	1.66
02	16.5	11.9	1.39
03	7.6	5.5	1.38
04	7.6	5.7	1.33
05	15.8	13.2	1.20
06	16.4	14.1	1.16
07	15.8	11.2	1.41
08	17.2	9.0	1.91
09	18.8	12.6	1.49
10	16.7	12.1	1.38
11	16.0	13.5	1.19
12	16.1	11.5	1.40
13	17.0	10.4	1.63
14	16.5	9.7	1.70
15	16.8	9.5	1.77
16	16.5	10.4	1.59
17	16.3	11.1	1.47
18	16.8	9.7	1.73
19	16.4	10.2	1.61
20	18.7	9.9	1.89
21	16.7	12.6	1.33
22	16.9	12.9	1.31
23	16.3	11.1	1.47
24	16.9	11.7	1.44
25	16.3	11.3	1.44
26	16.5	9.9	1.67
27	15.6	10.4	1.50

VIAL C continued...

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
28	17.0	10.2	1.67
29	17.3	11.6	1.49
30	16.3	10.9	1.50
31	16.1	9.9	1.63
32	19.1	10.6	1.80
33	17.0	9.5	1.79
34	16.8	11.1	1.51
35	16.8	9.7	1.73
36	17.7	10.9	1.62
37	17.8	11.5	1.55
38	16.5	9.7	1.70
39	17.9	12.2	1.47
40	16.5	9.9	1.67
41	15.6	10.7	1.46
42	15.6	10.2	1.53
43	16.5	9.5	1.74
44	16.8	13.6	1.24
45	15.6	11.6	1.34
46	16.3	9.7	1.68
47	16.5	10.4	1.59
48	18.0	9.9	1.82
49	17.2	10.7	1.61
50	17.7	13.2	1.34
51	17.9	12.0	1.49
52	16.7	11.7	1.43
53	17.1	11.5	1.49
54	16.5	12.9	1.28

Appendix IV continued...

VIAL D

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	17.2	12.2	1.42
02	17.7	11.0	1.60
03	16.8	12.6	1.33
04	17.0	12.9	1.32
05	15.4	10.8	1.43
06	17.9	11.5	1.56
07	15.6	9.6	1.62
08	16.8	11.5	1.46
09	15.8	10.3	1.53
10	15.6	10.6	1.48
11	15.4	9.6	1.60
12	16.1	10.6	1.52
13	15.8	11.2	1.41
14	16.8	9.9	1.70
15	15.8	9.4	1.68
16	15.8	9.2	1.72
17	18.0	12.3	1.46
18	18.4	12.1	1.53
19	16.5	11.6	1.43
20	17.7	11.8	1.50
21	16.1	9.7	1.66
22	18.4	11.3	1.63
23	17.3	11.1	1.56
24	18.2	11.6	1.57
25	16.8	9.7	1.73
26	15.1	10.6	1.42
27	17.7	10.2	1.74
28	17.3	10.2	1.70
29	16.8	10.9	1.54
30	16.5	10.6	1.56
31	17.5	10.4	1.68
32	15.8	10.9	1.45
33	17.7	11.3	1.57
34	16.5	11.1	1.49
35	16.1	10.2	1.58
36	15.4	11.8	1.30
37	17.5	11.1	1.57
38	17.5	11.1	1.57
39	17.3	9.7	1.78
40	16.8	10.9	1.54
41	18.0	11.1	1.62
42	16.1	9.9	1.63

VIAL E

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	17.3	11.1	1.56
02	18.0	12.1	1.50
03	17.0	11.1	1.53
04	18.4	10.4	1.77
05	17.0	11.8	1.44
06	17.5	11.8	1.48
07	15.6	10.9	1.43
08	17.5	11.3	1.55
09	19.4	11.1	1.75
10	17.7	12.1	1.46
11	17.0	11.1	1.53
12	18.7	12.3	1.52
13	17.3	10.9	1.59
14	15.4	11.3	1.36
15	14.2	11.3	1.26
16	16.5	11.8	1.40
17	15.1	12.3	1.23
18	17.7	10.2	1.74
19	13.9	10.6	1.31
20	18.0	11.1	1.62
21	16.1	12.1	1.33
22	17.0	10.4	1.63
23	17.7	13.0	1.36
24	16.5	11.6	1.42
25	15.1	10.9	1.39
26	16.8	9.9	1.70
27	16.3	10.9	1.50
28	15.6	11.1	1.41
29	17.3	11.3	1.53
30	16.6	11.4	1.46
31	18.6	11.2	1.66

Appendix IV continued...

VIAL F

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	15.8	11.5	1.37
02	16.8	10.3	1.63
03	16.3	11.0	1.48
04	14.7	11.0	1.34
05	16.1	11.2	1.44
06	17.7	12.1	1.46
07	18.4	11.8	1.56
08	17.5	11.8	1.48
09	17.0	10.9	1.56
10	15.8	12.1	1.31
11	17.7	11.6	1.53
12	17.5	11.6	1.51
13	17.7	11.3	1.57
14	17.5	11.1	1.58
15	17.7	11.3	1.57
16	18.2	13.0	1.40
17	18.7	10.9	1.72
18	17.7	11.6	1.53
19	18.9	10.9	1.73
20	16.8	11.8	1.42
21	20.6	13.5	1.53
22	19.4	12.5	1.55
23	18.7	11.3	1.65
24	16.8	11.3	1.49
25	14.4	11.6	1.24

VIAL F continued...

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
26	16.3	9.7	1.68
27	15.1	10.2	1.48
28	13.5	10.2	1.32
29	16.1	11.8	1.36
30	15.4	11.3	1.36
31	15.1	10.4	1.45
32	16.3	10.6	1.54
33	16.8	12.1	1.39
34	16.1	10.2	1.58
35	15.1	11.3	1.34
36	16.5	10.9	1.51
37	15.6	10.9	1.43
38	14.7	12.5	1.18
39	6.6	5.2	1.27
40	16.5	9.7	1.70
41	14.4	11.1	1.30
42	17.3	10.6	1.63
43	14.2	9.9	1.43
44	16.3	10.9	1.50
45	16.3	10.9	1.50
46	15.8	11.3	1.40
47	15.4	11.6	1.33
48	16.1	10.2	1.58
49	16.3	9.7	1.68

Appendix IV continued...

VIAL G

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	17.3	11.1	1.56
02	17.7	12.1	1.46
03	17.3	10.9	1.59
04	18.2	12.1	1.50
05	17.5	10.9	1.61
06	18.0	11.3	1.59
07	16.5	10.6	1.56
08	17.0	10.6	1.60
09	17.7	10.6	1.67
10	17.3	10.4	1.66
11	17.0	12.3	1.38
12	17.0	12.1	1.40
13	17.7	9.9	1.97
14	17.3	11.1	1.56
15	19.9	11.3	1.76
16	18.0	12.8	1.41
17	17.3	10.6	1.63
18	17.7	11.1	1.59
19	19.4	11.1	1.75
20	16.3	10.2	1.60
21	15.4	8.5	1.81
22	17.0	10.9	1.56
23	15.6	9.7	1.61
24	13.7	11.1	1.23
25	16.3	9.5	1.72
26	15.6	9.2	1.70
27	17.0	10.6	1.60
28	17.8	11.0	1.62
29	17.6	13.7	1.28
30	17.6	11.0	1.60
31	16.6	9.7	1.71
32	14.5	11.8	1.23

VIAL H

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	8.5	6.7	1.27
02	15.6	11.9	1.31
03	17.4	11.9	1.46
04	14.2	11.7	1.21
05	17.9	11.5	1.56
06	11.2	9.6	1.17
07	16.5	11.7	1.41
08	14.7	10.8	1.36
09	15.8	11.5	1.37
10	15.4	10.8	1.43
11	14.2	9.6	1.48
12	16.8	11.5	1.46
13	15.6	11.2	1.39
14	15.6	10.6	1.47
15	16.5	11.5	1.43
16	17.2	11.0	1.56
17	15.6	11.5	1.36
18	15.4	11.0	1.40
19	17.0	12.3	1.38
20	17.7	12.1	1.46
21	16.5	10.9	1.51
22	15.8	11.6	1.36
23	12.1	9.7	1.25
24	18.2	10.9	1.67
25	10.2	6.6	1.55
26	13.9	10.9	1.28
27	7.1	5.9	1.20
28	8.3	5.2	1.60
29	5.4	3.3	1.64
30	19.1	12.1	1.58
31	17.5	11.8	1.48
32	18.7	11.8	1.58
33	13.7	9.7	1.41
34	10.9	9.7	1.12
35	16.1	11.8	1.36
36	18.4	11.6	1.59
37	16.5	9.9	1.67
38	16.3	9.5	1.72
39	13.5	10.4	1.30
40	16.1	11.3	1.42
41	16.3	10.6	1.54
42	16.1	10.9	1.48
43	15.1	11.1	1.36
44	17.3	10.6	1.63
45	14.9	9.7	1.54
46	11.3	10.4	1.09
47	16.8	10.9	1.54
48	18.9	10.9	1.73

Appendix IV continued...

VIAL I

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	16.8	9.9	1.70
02	12.2	9.0	1.36
03	7.6	5.7	1.33
04	13.8	10.6	1.30
05	14.9	9.9	1.51
06	6.4	6.0	1.07
07	6.4	5.7	1.12
08	5.3	5.3	1.00
09	8.0	5.5	1.45
10	11.0	9.4	1.17
11	6.0	4.8	1.25
12	9.0	5.7	1.58
13	6.9	4.4	1.57
14	6.0	5.5	1.09
15	5.2	5.0	1.04
16	7.3	6.9	1.06
17	6.1	5.0	1.22
18	5.0	4.7	1.06
19	8.0	5.9	1.36
20	16.5	10.4	1.59
21	15.4	10.2	1.51
22	5.7	5.4	1.56
23	14.9	10.2	1.46
24	14.9	10.2	1.46
25	16.3	9.7	1.68
26	13.7	9.7	1.41
27	15.4	9.5	1.62
28	15.1	9.7	1.56
29	14.4	9.5	1.52
30	16.1	9.9	1.63
31	16.1	9.9	1.63
32	14.4	10.4	1.38
33	15.1	10.2	1.48
34	16.3	9.7	1.68
35	14.9	11.0	1.35
36	17.8	11.2	1.59
37	7.5	6.8	1.10
38	12.6	10.1	1.25
39	15.5	11.4	1.36
40	14.5	11.8	1.23
41	13.6	11.0	1.24
42	17.4	11.6	1.50
43	12.6	9.1	1.38
44	8.7	7.9	1.10
45	14.3	10.3	1.39
46	12.0	11.2	1.07
47	13.7	11.4	1.20

VIAL J

NO.	LENGTH (u)	WIDTH (u)	L:W RATIO
01	8.0	5.7	1.40
02	16.8	10.3	1.63
03	14.9	10.6	1.41
04	13.3	10.3	1.29
05	15.8	8.5	1.86
06	16.1	9.9	1.63
07	18.4	11.1	1.66
08	16.3	10.6	1.54
09	7.6	5.4	1.41
10	14.4	10.2	1.41
11	13.7	8.3	1.65
12	11.8	9.3	1.27

Appendix V. Relative fluidity values of hemoglobin isomorphs, determined through comparison with human Hb A standard. The C stands for cathodal bands while the A represents anodal bands. Dashed lines represent bands which were not visualized on that particular electrophoretic plate.

VIAL C

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	1.11	1.03	0.92	0.85	0.79	0.70	0.17	0.15	0.29	0.47	0.56
02	1.11	1.01	0.91	0.84	0.76	0.68	0.17	0.16	0.29	0.48	0.55
03	1.12	1.04	0.93	0.85	0.78	0.68	0.14	0.18	0.31	0.52	0.58
04	1.12	1.01	0.90	0.82	0.77	0.66	0.13	0.19	0.30	0.52	0.60
05	1.10	1.03	0.91	0.84	0.77	0.64	0.17	0.15	0.30	0.47	0.56
06	1.10	1.01	0.90	0.83	0.76	0.64	0.17	0.14	0.29	0.45	0.56
07	1.08	1.02	0.90	0.83	0.76	0.64	0.16	0.14	0.28	0.46	0.59
08	1.10	1.03	0.92	0.84	0.77	0.65	0.17	0.14	0.28	0.45	0.57
09	1.12	1.04	0.94	0.85	0.78	0.71	0.17	0.20	0.34	0.51	--
10	1.11	1.03	0.93	0.86	0.77	0.70	0.16	0.20	0.35	0.51	--
11	1.11	1.02	0.92	0.84	0.77	0.68	0.16	0.19	0.33	0.51	--
12	1.07	1.01	0.90	0.83	0.76	0.67	0.17	0.19	0.32	0.52	--
13	1.08	1.00	0.90	0.83	0.75	0.68	0.12	0.21	0.35	0.49	0.60
14	1.06	0.98	0.88	0.81	0.73	0.67	0.11	0.20	0.35	0.50	0.60
15	1.09	1.00	0.89	0.82	0.74	0.67	0.12	0.20	0.35	0.51	0.58
16	1.07	1.00	0.89	0.81	0.74	0.66	0.12	0.20	0.34	0.52	--
17	1.06	0.99	0.91	0.86	0.76	0.65	0.17	0.13	0.28	0.45	0.61
18	1.06	1.00	0.90	0.85	0.75	0.65	0.18	0.12	0.29	0.45	0.59
19	1.13	1.03	0.91	0.87	0.77	0.62	0.15	0.14	0.26	0.46	0.58
20	1.12	1.03	0.92	0.86	0.77	0.63	0.16	0.12	0.26	0.45	0.58
n	20	20	20	20	20	20	20	20	20	20	15
$\bar{X}$	1.10	1.02	0.91	0.84	0.76	0.66	0.15	0.17	0.31	0.49	0.58
SE	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01
95% CI	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Appendix V continued...

VIAL D

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	1.10	1.03	0.91	0.85	0.77	0.70	0.18	0.14	0.26	0.46	0.57
02	1.07	1.01	0.92	0.85	0.77	0.70	0.18	0.14	0.27	0.45	0.58
03	1.06	0.99	0.90	0.82	0.76	0.68	0.19	0.12	0.25	0.42	0.58
04	1.10	1.02	0.92	0.84	0.77	0.70	0.19	0.13	0.28	0.44	0.60
05	1.08	1.00	0.90	0.82	0.76	0.69	0.17	0.16	0.29	0.49	0.62
06	1.06	0.99	0.89	0.82	0.74	0.68	0.17	0.17	0.29	0.49	0.58
07	1.10	1.03	0.91	0.82	0.76	0.68	0.15	0.19	0.33	0.52	0.55
08	1.08	1.01	0.92	0.82	0.76	0.69	0.15	0.19	0.32	0.52	0.55
09	1.10	1.01	0.91	0.85	0.78	0.66	0.15	0.20	0.34	0.47	--
10	1.08	1.00	0.91	0.83	0.75	0.65	0.15	0.21	0.35	0.47	--
11	1.12	1.01	0.92	0.86	0.79	0.69	0.15	0.21	0.35	0.48	0.57
12	1.10	1.02	0.91	0.84	0.76	0.66	0.15	0.21	0.34	0.45	--
13	1.10	1.03	0.93	0.84	0.78	0.68	0.14	0.20	0.34	0.52	0.58
14	1.07	1.02	0.90	0.82	0.76	0.66	0.14	0.21	0.35	0.52	0.59
15	1.10	1.03	0.90	0.82	0.74	0.67	0.13	0.21	0.35	0.52	0.55
16	1.06	1.00	0.88	0.80	0.75	0.66	0.14	0.19	0.33	0.51	0.55
17	1.08	1.04	0.93	0.86	0.79	--	0.22	0.11	0.26	0.45	--
18	1.06	0.99	0.90	0.83	0.76	--	0.22	0.10	0.25	0.44	--
19	1.07	1.02	0.91	0.85	0.79	0.66	0.22	0.10	0.28	0.43	--
20	1.06	0.99	0.89	0.84	0.77	0.65	0.21	0.10	0.27	0.43	--
21	1.08	1.01	0.89	0.82	0.75	0.68	0.11	0.22	0.35	0.49	0.57
22	1.06	1.00	0.89	0.82	0.75	0.69	0.12	0.21	0.35	0.51	0.58
23	1.10	1.03	0.92	0.84	0.75	0.71	0.12	0.22	0.35	0.48	0.57
24	1.09	1.02	0.91	0.84	0.76	0.71	0.12	0.20	0.35	0.48	0.58
25	1.07	0.98	0.90	0.85	0.77	0.70	0.16	0.13	0.33	0.51	0.57
26	1.06	0.98	0.88	0.83	0.76	0.68	0.14	0.13	0.31	0.49	0.57
27	1.11	1.00	0.91	0.81	0.74	0.66	0.15	0.15	0.29	0.48	0.55
28	1.10	1.01	0.91	0.81	0.74	0.67	0.15	0.15	0.30	0.48	0.56
29	1.08	1.00	0.93	0.84	0.75	0.63	0.20	0.17	0.27	0.46	0.56
30	1.08	0.98	0.90	0.83	0.74	0.64	0.19	0.16	0.27	0.46	0.57
31	1.13	0.98	0.91	0.86	0.74	0.65	0.20	0.17	0.26	0.48	0.60
32	1.11	0.97	0.90	0.84	0.73	0.64	0.21	0.17	0.25	0.49	--
33	1.12	1.02	0.92	0.86	0.76	0.68	0.16	0.16	0.25	0.47	0.57
34	1.11	1.02	0.92	0.86	0.77	0.69	0.16	0.18	0.25	0.48	0.56
35	1.08	1.01	0.94	0.85	0.76	0.65	0.15	0.16	0.28	0.51	0.58
36	1.06	1.01	0.91	0.86	0.74	0.65	0.14	0.16	0.29	0.51	0.57
n	36	36	36	36	36	34	36	36	36	36	28
$\bar{X}$	1.09	1.01	0.91	0.84	0.76	0.67	0.16	0.17	0.30	0.48	0.57
SE	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.00
95% CI	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Appendix V continued...

VIAL E

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	1.09	1.02	0.92	0.84	0.78	0.70	0.21	0.13	0.24	0.44	0.57
02	1.12	1.04	0.94	0.86	0.79	0.70	0.17	0.15	0.28	0.49	0.57
03	1.08	1.03	0.93	0.86	0.79	0.72	0.20	0.13	0.27	0.46	0.58
04	1.11	1.03	0.94	0.86	0.79	0.71	0.18	0.17	0.31	0.50	0.55
05	1.11	1.00	0.94	0.86	0.78	0.72	0.14	0.20	0.33	0.50	--
06	1.08	1.02	0.91	0.82	0.75	0.70	0.13	0.19	0.32	0.51	--
07	1.11	1.04	0.92	0.84	0.75	0.70	0.14	0.18	0.33	0.51	--
08	1.11	1.03	0.91	0.83	0.75	0.69	0.15	0.18	0.33	0.53	--
09	1.08	1.00	0.90	0.82	0.72	0.66	0.12	0.20	0.35	0.51	0.60
10	1.05	0.99	0.88	0.81	0.73	0.66	0.11	0.21	0.35	0.52	0.59
11	1.07	0.98	0.89	0.80	0.72	0.64	0.12	0.21	0.35	0.51	0.60
12	1.07	0.98	0.89	0.81	0.72	0.66	0.12	0.21	0.34	0.50	0.59
13	1.08	0.99	0.89	0.80	0.75	0.67	0.19	0.11	0.25	0.42	0.54
14	1.06	0.98	0.89	0.82	0.75	0.68	0.19	0.12	0.25	0.44	0.55
15	1.08	1.01	0.91	0.85	0.76	0.70	0.20	0.13	0.28	0.46	0.57
16	1.09	1.02	0.92	0.85	0.77	0.72	0.20	0.14	0.28	0.45	0.55
17	1.06	0.99	0.89	0.82	0.76	0.70	0.18	0.13	0.28	0.46	--
18	1.07	1.01	0.90	0.84	0.77	--	0.18	0.12	0.28	0.44	--
19	1.12	1.05	0.94	0.86	0.78	--	0.20	0.13	0.29	0.47	--
20	1.07	1.02	0.90	0.83	0.77	--	0.19	0.14	0.29	0.47	--
21	1.08	1.00	0.89	0.82	0.75	0.69	0.12	0.21	0.37	0.45	0.58
22	1.07	1.00	0.88	0.81	0.73	0.68	0.13	0.20	0.35	0.47	0.56
23	1.10	1.01	0.93	0.83	0.76	0.69	0.12	0.22	0.37	0.47	0.62
24	1.07	1.00	0.88	0.80	0.74	0.68	0.12	0.22	0.37	0.50	0.60
25	1.08	0.97	0.93	0.84	0.74	0.68	0.17	0.17	0.32	0.50	0.60
26	1.08	0.98	0.94	0.84	0.75	0.68	0.15	0.18	0.31	0.50	0.61
27	1.11	0.99	0.90	0.84	0.74	0.68	0.16	0.18	0.29	0.48	0.58
28	1.10	0.99	0.89	0.84	0.72	0.66	0.16	0.17	0.29	0.48	0.58
29	1.09	1.01	0.92	0.87	0.74	0.65	0.16	0.16	0.29	0.50	--
30	1.10	1.00	0.91	0.85	0.75	0.68	0.17	0.18	0.29	0.49	--
31	1.09	1.00	0.89	0.84	0.73	0.63	0.13	0.15	0.30	0.52	0.60
32	1.09	0.98	0.90	0.83	0.73	0.64	0.16	0.17	0.28	0.50	0.60
33	1.06	1.02	0.91	0.86	0.75	0.64	0.17	0.16	0.30	0.46	0.57
34	1.06	1.01	0.91	0.85	0.74	0.64	0.15	0.15	0.30	0.46	0.56
n	34	34	34	34	34	31	34	34	34	34	24
$\bar{X}$	1.09	1.01	0.91	0.84	0.75	0.68	0.16	0.17	0.31	0.48	0.58
SE	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00
95% CI	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Appendix V continued...

VIAL F

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	1.11	1.04	0.93	0.86	0.78	0.69	0.17	0.16	0.29	0.50	--
02	1.11	1.02	0.93	0.85	0.77	0.68	0.14	0.17	0.30	0.50	--
03	1.11	1.04	0.94	0.87	0.79	0.72	0.19	0.14	0.31	0.48	--
04	1.10	1.02	0.93	0.85	0.79	0.71	0.18	0.14	0.28	0.47	--
05	1.06	1.00	0.89	0.83	0.75	--	0.18	0.14	0.29	0.46	--
06	1.07	1.00	0.89	0.83	0.75	--	0.17	0.15	0.28	0.46	--
07	1.11	1.03	0.92	0.84	0.77	--	0.18	0.15	0.32	0.48	--
08	1.10	1.04	0.91	0.83	0.75	--	0.17	0.15	0.30	0.49	--
09	1.12	1.04	0.91	0.82	0.75	0.67	0.14	0.19	0.36	0.49	0.57
10	1.07	0.99	0.90	0.83	0.75	0.66	0.12	0.19	0.34	0.49	0.55
11	1.08	0.99	0.89	0.81	0.74	0.65	0.12	0.18	0.33	0.49	0.54
12	1.07	0.99	0.88	0.81	0.72	0.65	0.13	0.18	0.33	0.48	0.54
13	1.12	1.01	0.89	0.82	0.73	0.66	0.18	0.12	0.28	0.44	0.56
14	1.06	1.00	0.89	0.81	0.73	0.67	0.18	0.13	0.27	0.44	0.56
15	1.08	1.01	0.91	0.84	0.75	0.68	0.18	0.14	0.28	0.47	0.56
16	1.07	1.00	0.91	0.83	0.74	0.69	0.20	0.13	0.29	0.45	0.55
17	1.06	1.00	0.89	0.83	0.77	--	0.19	0.13	0.26	0.45	--
18	1.04	0.98	0.88	0.83	0.75	--	0.18	0.13	0.26	0.44	--
19	1.10	1.04	0.93	0.87	0.79	--	0.19	0.13	0.29	0.46	--
20	1.08	1.01	0.92	0.82	0.77	--	0.18	0.14	0.29	0.47	--
21	1.08	1.01	0.91	0.83	0.75	--	0.12	0.20	0.37	0.48	--
22	1.09	1.00	0.91	0.83	0.76	--	0.13	0.22	0.35	0.46	--
23	1.09	1.01	0.91	0.82	0.76	0.69	0.11	0.20	0.37	0.52	--
24	1.08	1.00	0.90	0.83	0.76	0.69	0.11	0.20	0.36	0.51	--
25	1.12	1.04	0.91	0.86	0.76	0.69	0.16	0.15	0.31	0.49	0.56
26	1.09	1.02	0.90	0.85	0.77	0.71	0.15	0.13	0.31	0.50	0.56
27	1.08	1.02	0.92	0.82	0.77	0.64	0.16	0.15	0.27	0.46	0.55
28	1.09	1.01	0.90	0.81	0.76	0.65	0.13	0.14	0.28	0.47	0.56
29	1.12	1.01	0.93	0.85	0.75	0.66	0.14	0.14	0.24	0.45	0.56
30	1.10	0.98	0.91	0.83	0.75	0.66	0.14	0.15	0.26	0.47	0.57
31	1.07	1.00	0.92	0.86	0.74	0.66	0.12	0.13	0.26	0.47	0.59
32	1.06	0.98	0.92	0.85	0.74	0.67	0.13	0.14	0.27	0.46	0.59
33	1.10	0.99	0.90	0.83	0.74	0.67	0.14	0.12	0.27	0.49	0.58
34	1.09	0.97	0.88	0.80	0.75	0.68	0.14	0.14	0.27	0.51	0.58
35	1.10	0.99	0.94	0.85	0.76	0.66	0.15	0.15	0.26	0.49	0.61
36	1.11	1.00	0.93	0.84	0.74	0.68	0.17	0.15	0.26	0.50	0.59
37	1.12	1.04	0.93	0.87	0.76	0.69	0.18	0.16	0.26	0.48	0.57
38	1.11	1.03	0.92	0.85	0.77	0.70	0.19	0.17	0.25	0.49	0.57
39	1.09	1.00	0.90	0.82	0.75	0.69	0.16	0.19	0.25	0.48	0.57
40	1.07	1.01	0.90	0.84	0.76	0.66	0.15	0.19	0.26	0.46	0.57
n	40	40	40	40	40	30	40	40	40	40	24
$\bar{X}$	1.09	1.01	0.91	0.84	0.76	0.68	0.16	0.16	0.29	0.48	0.57
SE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00
95% CI	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Appendix V continued...

VIAL G

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	1.09	1.02	0.92	0.84	0.77	--	0.17	0.16	--	0.49	--
02	1.09	1.01	0.92	0.84	0.76	--	0.16	0.16	--	0.49	--
03	1.10	1.03	0.92	0.85	0.76	--	0.16	0.17	--	0.49	--
04	1.08	1.01	0.91	0.84	0.76	--	0.16	0.16	--	0.49	--
05	1.10	1.02	0.91	0.83	0.76	0.69	0.15	0.20	0.33	0.52	0.61
06	1.06	0.99	0.89	0.81	0.75	0.67	0.14	0.19	0.33	0.50	0.59
07	1.11	1.04	0.93	0.84	0.75	0.69	0.17	0.14	0.26	0.45	0.59
08	1.10	1.04	0.92	0.83	0.76	0.68	0.17	0.13	0.26	0.46	0.58
09	1.10	1.01	0.90	0.83	0.78	0.72	0.14	0.20	--	0.51	--
10	1.07	1.00	0.90	0.81	0.77	0.71	0.14	0.21	--	0.49	--
11	1.10	1.00	0.92	0.84	0.77	--	0.14	0.18	--	0.53	--
12	1.08	1.00	0.89	0.83	0.75	--	0.14	0.18	--	0.52	--
13	1.11	1.04	0.93	0.86	0.80	0.72	0.22	0.09	0.27	0.46	--
14	1.06	1.01	0.91	0.84	0.79	0.71	0.22	0.10	0.29	0.46	--
15	1.06	0.99	0.89	0.81	0.76	0.70	0.22	0.11	0.26	0.43	--
16	1.08	0.97	0.88	0.81	0.74	0.70	0.22	0.10	0.27	0.45	--
17	1.09	1.02	0.91	0.84	0.76	--	0.17	0.13	0.27	0.45	--
18	1.07	1.00	0.89	0.82	0.74	--	0.16	0.15	0.25	0.46	--
19	1.06	1.00	0.91	0.83	0.76	--	0.20	0.10	0.25	0.44	--
20	1.07	1.00	0.89	0.83	0.77	--	0.21	0.10	0.25	0.44	--
21	1.08	1.00	0.90	0.83	0.76	--	0.12	0.20	0.35	0.50	--
22	1.07	1.00	0.87	0.81	0.74	--	0.11	0.21	0.36	0.51	--
23	1.07	1.00	0.90	0.82	0.76	--	0.13	0.19	0.34	0.52	--
24	1.06	1.00	0.90	0.82	0.75	--	0.12	0.19	0.35	0.51	--
25	1.07	1.00	0.91	0.83	0.77	0.70	0.17	0.14	0.28	0.51	0.59
26	1.06	1.01	0.89	0.82	0.75	0.67	0.14	0.13	0.27	0.50	0.60
27	1.07	0.98	0.89	0.81	0.75	0.68	0.14	0.17	0.27	0.48	0.59
28	1.07	0.98	0.86	0.81	0.73	0.66	0.14	0.16	0.26	0.45	0.60
29	1.05	0.98	0.89	0.84	0.73	0.63	0.13	0.19	0.30	0.46	0.60
30	1.06	0.99	0.91	0.83	0.72	0.62	0.11	0.17	0.31	0.46	0.58
31	1.11	1.02	0.93	0.83	0.73	0.63	0.14	0.17	0.29	0.43	0.55
32	1.11	1.04	0.94	0.83	0.73	0.63	0.14	0.17	0.31	0.45	0.55
33	1.07	0.99	0.92	0.83	0.77	--	0.18	0.14	--	0.49	--
34	1.09	0.98	0.92	0.83	0.77	--	0.18	0.14	--	0.49	--
35	1.08	1.01	0.92	0.84	0.76	0.65	0.17	0.16	0.29	0.49	0.59
36	1.09	0.99	0.91	0.82	0.74	0.66	0.17	0.15	0.30	0.50	0.59
n	36	36	36	36	36	20	36	36	26	36	14
$\bar{X}$	1.08	1.01	0.91	0.83	0.76	0.68	0.16	0.16	0.29	0.48	0.59
SE	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01
95% CI	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Appendix V continued...

VIAL H

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	1.09	1.02	0.92	0.85	0.77	--	0.20	0.14	--	0.47	--
02	1.10	1.02	0.92	0.86	0.79	--	0.20	0.13	--	0.45	--
03	1.10	1.03	0.93	0.86	0.79	--	0.17	0.15	--	0.49	--
04	1.11	1.04	0.93	0.86	0.78	--	0.16	0.17	--	0.52	--
05	1.10	1.03	0.93	0.85	0.79	--	0.22	0.11	--	0.42	--
06	1.07	1.01	0.90	0.83	0.76	--	0.23	0.10	--	0.41	--
07	1.11	1.03	0.94	0.86	0.79	--	0.16	0.21	--	0.51	--
08	1.07	1.01	0.91	0.85	0.77	--	0.17	0.21	--	0.49	--
09	1.10	1.04	0.93	0.87	0.80	--	0.19	0.12	--	0.44	--
10	1.06	1.00	0.91	0.84	0.76	--	0.18	0.12	--	0.42	--
11	1.10	1.01	0.92	0.86	0.79	--	0.21	0.12	--	0.43	--
12	1.08	1.01	0.92	0.86	0.79	--	0.21	0.13	--	0.44	--
13	1.09	1.03	0.92	0.86	0.79	--	0.21	0.09	0.26	0.47	--
14	1.05	0.99	0.89	0.82	0.76	--	0.19	0.10	0.27	0.46	--
15	1.09	1.02	0.92	0.83	0.76	--	0.18	0.13	0.29	0.45	--
16	1.05	0.98	0.87	0.80	0.73	--	0.17	0.13	0.27	0.43	--
17	1.09	1.00	0.90	0.83	0.75	--	0.12	0.22	0.35	0.50	--
18	1.06	1.00	0.88	0.81	0.74	--	0.11	0.22	0.36	0.50	--
19	1.10	0.99	0.87	0.82	0.78	--	0.14	0.20	0.35	0.47	--
20	1.07	0.99	0.88	0.82	0.74	--	0.13	0.20	0.35	0.47	--
21	1.11	0.99	0.92	0.83	0.74	--	0.13	0.17	0.30	0.46	0.58
22	1.12	1.03	0.91	0.83	0.76	0.67	0.12	0.16	0.30	0.46	0.56
23	1.09	1.02	0.89	0.82	0.75	--	0.13	0.15	--	0.48	--
24	1.09	1.02	0.91	0.84	0.75	0.70	0.14	0.15	--	0.48	--
25	1.09	1.03	0.90	0.85	0.76	--	0.18	0.16	0.32	0.47	--
26	1.08	1.03	0.89	0.83	0.77	--	0.17	0.16	0.29	0.48	--
27	1.07	1.01	0.88	0.83	0.76	--	0.20	0.16	0.31	0.44	--
28	1.06	1.00	0.89	0.83	0.75	--	0.18	0.16	0.32	0.44	--
29	1.07	1.04	0.93	0.86	0.76	--	0.14	0.18	--	0.51	--
30	1.05	1.03	0.92	0.86	0.76	--	0.15	0.17	--	0.50	--
31	1.07	1.01	0.93	0.87	0.75	--	0.12	0.20	0.31	0.47	--
32	1.07	1.03	0.94	0.87	0.77	--	0.14	0.17	0.32	0.46	--
n	32	32	32	32	32	2	32	32	16	32	2
$\overline{X}$	1.08	1.02	0.91	0.84	0.77	0.69	0.17	0.16	0.31	0.47	0.57
SE	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.01	0.01	0.01	0.01
95% CI	0.01	0.01	0.01	0.01	0.01	0.03	0.01	0.01	0.01	0.01	0.02

Appendix V continued...

VIAL I

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	1.07	1.00	0.90	0.82	0.76	--	0.15	0.20	--	0.48	--
02	1.10	1.03	0.92	0.84	0.78	--	0.14	0.20	--	0.49	--
03	1.08	0.99	0.89	0.82	0.74	--	0.12	0.23	--	0.46	--
04	1.07	0.99	0.88	0.81	0.75	--	0.13	0.22	--	0.48	--
05	1.07	1.01	0.88	0.82	0.76	--	0.14	0.20	--	0.48	--
06	1.06	1.00	0.89	0.82	0.76	--	0.14	0.20	--	0.49	--
07	1.07	0.98	0.92	0.83	0.76	--	0.18	0.14	--	0.47	--
08	1.06	1.00	0.94	0.83	0.77	--	0.13	0.12	--	0.48	--
09	1.07	1.03	0.92	0.81	0.75	--	0.13	0.14	--	0.48	--
10	1.10	1.03	0.89	0.82	0.75	--	0.14	0.13	--	0.48	--
11	1.08	1.02	0.92	0.88	0.74	--	0.15	0.19	--	0.45	--
12	1.10	1.04	0.92	0.86	0.75	--	0.15	0.19	--	0.44	--
13	1.08	1.00	0.91	0.86	0.75	--	0.17	0.17	--	0.44	--
14	1.11	1.02	0.92	0.85	0.73	--	0.18	0.19	--	0.43	--
15	1.06	0.98	0.92	0.86	0.75	--	0.16	0.14	--	0.46	--
16	1.08	1.01	0.93	0.86	0.74	--	0.18	0.13	0.27	0.46	--
17	1.08	1.00	0.89	0.86	0.77	--	0.16	0.17	--	0.47	--
18	1.09	1.02	0.92	0.86	0.75	--	0.17	0.17	--	0.44	--
n	18	18	18	18	18	0	18	18	1	18	0
$\bar{X}$	1.08	1.01	0.91	0.84	0.75		0.15	0.17	0.27	0.47	
SE	0.00	0.00	0.00	0.01	0.00		0.00	0.01		0.00	
95% CI	0.01	0.01	0.01	0.01	0.01		0.01	0.01		0.01	

Appendix VI. Mean relative hemoglobin abundancy of cathodal and anodal bands as determined from densitometric tracings of cellulose acetate strips. In instances where not all bands were present, a value of 0 was ascribed to the missing band. Values are percent values, i.e. the total of all isomorphs in each run number is 100%.

VIAL C

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	06.0	09.5	09.7	26.4	19.0	01.5	00.9	10.4	00.3	16.1	00.3
02	06.0	10.4	10.1	26.7	18.3	02.0	00.5	09.0	00.4	15.2	00.5
03	04.4	08.7	08.7	27.8	19.0	01.0	01.4	11.6	00.8	16.1	00.4
04	04.8	08.9	09.2	31.1	18.7	00.4	00.8	10.7	01.0	13.9	00.5
05	07.7	07.8	08.3	29.4	17.6	00.0	01.0	13.1	00.2	14.9	00.0
06	07.4	07.8	08.6	32.5	17.5	00.0	00.8	11.2	00.0	14.2	00.0
07	07.7	07.5	08.6	32.0	16.6	00.0	01.1	12.0	00.3	14.2	00.0
08	07.5	08.6	08.5	32.3	18.2	00.0	00.7	10.5	00.0	13.8	00.0
09	07.0	07.9	09.6	31.5	19.5	00.3	01.2	09.2	00.3	13.5	00.0
10	07.3	07.1	12.2	31.5	18.4	00.0	01.2	08.9	00.2	13.2	00.0
11	07.5	07.3	10.3	34.0	19.0	00.7	01.1	08.1	00.2	11.6	00.2
12	07.0	07.4	12.0	29.7	18.8	00.5	01.3	09.6	00.2	13.5	00.0
13	07.4	06.7	09.0	30.2	18.1	00.4	01.1	10.4	00.9	15.6	00.2
14	07.6	06.9	08.1	30.3	18.1	00.3	01.0	10.6	00.9	15.9	00.3
15	07.6	06.8	07.0	28.8	18.3	00.3	01.1	11.7	01.0	17.2	00.2
n	15	15	15	15	15	15	15	15	15	15	15
$\bar{X}$	06.9	08.0	09.3	30.3	18.3	00.5	01.0	10.5	00.4	14.6	00.2
SE	00.4	00.3	00.4	00.6	00.2	00.2	00.1	00.3	00.1	00.4	00.0
95% CI	00.5	00.5	00.6	01.0	00.3	00.3	00.1	00.6	00.2	00.7	00.1

Appendix VI continued...

VIAL D

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	06.5	09.3	09.7	25.2	19.4	01.3	01.6	10.9	00.1	16.0	00.1
02	07.0	10.2	09.3	27.0	17.1	01.8	01.8	10.8	00.4	14.3	00.3
03	04.6	08.8	09.1	28.5	19.5	00.2	00.5	11.5	00.2	16.9	00.1
04	05.4	09.0	08.9	30.1	18.0	00.2	00.3	11.3	00.3	16.2	00.2
05	08.5	10.2	09.9	26.3	18.2	00.0	01.2	12.4	00.0	13.3	00.0
06	09.8	10.7	10.6	28.4	17.7	00.0	00.8	10.9	00.0	11.1	00.0
07	08.5	10.9	10.4	27.4	17.1	00.1	00.5	11.1	00.0	14.0	00.0
08	08.7	09.8	09.7	27.0	17.7	00.0	00.7	12.6	00.0	13.8	00.0
09	07.3	08.0	07.8	27.4	19.9	00.2	01.4	12.1	00.1	15.8	00.0
10	07.2	07.4	07.6	30.0	18.5	00.0	01.1	12.1	00.0	16.0	00.0
11	07.4	07.4	07.5	28.0	20.6	00.0	00.6	12.5	00.0	16.1	00.0
12	07.8	08.3	07.8	28.9	18.2	00.0	00.8	13.1	00.4	14.7	00.0
13	07.4	08.1	11.4	32.2	19.2	00.0	01.0	09.0	00.0	11.7	00.0
14	06.5	07.8	10.5	32.7	19.9	00.0	00.6	09.7	00.0	12.3	00.0
15	07.0	07.6	09.6	33.5	19.8	00.2	01.0	08.8	00.4	12.1	00.0
16	06.4	07.7	10.3	34.6	21.5	00.2	01.2	07.6	00.2	10.3	00.0
17	05.0	07.7	12.9	29.1	14.1	00.4	01.1	12.1	00.2	17.0	00.4
18	05.6	07.7	12.9	29.8	14.1	00.2	01.6	11.1	00.3	16.5	00.2
19	05.2	07.9	11.4	30.0	14.9	00.4	00.9	12.6	00.3	16.2	00.2
20	06.6	07.5	13.1	30.4	15.3	00.4	01.2	11.2	00.3	13.9	00.1
21	04.6	05.9	11.4	26.4	16.7	00.3	01.4	13.7	01.0	18.5	00.2
22	04.9	06.0	11.5	26.0	16.3	00.2	02.2	13.7	01.0	18.3	00.3
23	04.5	06.0	11.6	26.1	16.4	00.2	02.2	13.8	01.0	18.3	00.3
24	04.4	05.5	10.1	27.0	16.5	00.2	01.9	13.6	01.0	19.2	00.6
25	06.4	07.9	07.7	31.0	18.4	00.3	01.6	09.4	00.7	17.3	00.3
26	05.9	07.2	08.0	30.2	17.7	00.3	00.7	11.8	00.7	17.2	00.3
27	06.4	07.4	07.6	29.8	17.4	00.3	00.8	11.8	00.8	17.2	00.5
28	05.8	07.1	07.7	30.1	16.7	00.3	01.2	11.9	00.7	18.1	00.4
29	05.9	07.1	07.7	30.2	16.9	00.1	01.2	11.8	00.7	18.0	00.4
n	29	29	29	29	29	29	29	29	29	29	29
$\bar{X}$	06.5	08.0	09.8	29.1	17.7	00.3	01.1	11.5	00.4	15.5	00.2
SE	00.3	00.3	00.3	00.4	00.3	00.1	00.1	00.3	00.1	00.5	00.0
95% CI	00.4	00.4	00.6	00.7	00.6	00.1	00.2	00.5	00.1	00.8	00.1

Appendix VI continued...

VIAL E

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	05.4	09.3	10.0	29.3	19.7	00.0	00.4	10.3	00.1	15.4	00.0
02	09.6	10.7	10.2	28.4	19.8	01.0	01.0	08.9	00.3	10.1	00.0
03	08.8	10.0	09.9	27.9	16.7	00.2	01.7	11.7	00.6	12.3	00.2
04	07.8	10.2	09.4	28.9	16.4	00.2	02.0	10.9	00.4	13.7	00.1
05	07.2	08.2	09.1	29.2	19.3	00.4	01.0	09.4	00.6	15.2	00.4
06	07.7	08.7	08.9	30.4	17.2	00.1	00.7	10.0	00.4	15.6	00.3
07	07.8	08.0	08.4	29.3	18.3	00.7	01.2	10.1	01.2	14.4	00.6
08	08.8	08.6	08.9	29.9	16.7	00.4	00.9	10.3	00.5	14.7	00.3
09	06.0	08.2	07.8	25.0	19.4	00.6	02.2	14.1	00.6	15.9	00.1
10	05.8	09.7	08.0	25.0	18.0	01.0	02.3	13.5	00.7	15.7	00.2
11	06.0	08.7	08.2	25.0	18.2	00.7	02.4	14.4	00.7	15.4	00.2
12	06.3	09.0	08.2	23.5	19.0	01.0	02.3	13.8	00.6	15.7	00.4
13	06.8	07.0	11.4	30.4	18.6	00.0	01.0	10.7	00.3	13.8	00.0
14	07.1	07.2	12.0	28.5	17.8	00.3	01.4	11.8	00.6	13.3	00.0
15	06.3	06.5	12.2	32.4	18.1	00.0	00.6	09.8	00.1	14.1	00.0
16	06.2	06.4	13.9	32.0	18.2	00.0	00.6	09.8	00.1	12.9	00.0
17	05.9	07.0	13.2	27.5	14.5	00.4	01.6	12.0	01.4	16.6	00.0
18	05.5	07.1	13.3	26.9	13.9	00.7	01.4	12.9	00.2	17.8	00.2
19	06.1	07.0	12.5	28.2	13.9	00.3	01.6	12.3	01.0	16.6	00.3
20	04.9	06.0	11.5	30.1	14.6	00.2	01.7	13.3	00.4	17.0	00.3
21	04.9	06.0	11.5	29.8	14.4	00.2	01.9	13.3	00.8	17.0	00.2
22	07.9	08.4	08.8	29.7	16.7	00.2	00.9	09.7	00.4	17.0	00.3
23	07.7	08.4	08.7	27.3	16.3	00.3	00.9	12.9	00.5	16.7	00.3
24	07.3	08.0	09.1	29.2	16.5	00.3	00.7	11.4	00.3	17.2	00.0
25	07.1	07.9	08.7	28.2	17.0	00.3	01.0	11.8	00.4	17.6	00.0
26	06.7	08.0	08.4	26.9	19.3	00.2	00.7	12.1	01.0	16.5	00.2
27	06.4	08.1	08.5	26.9	19.0	00.4	00.6	12.2	01.2	16.4	00.3
28	06.5	08.2	08.4	26.5	18.8	00.2	00.9	13.0	00.8	16.5	00.2
29	06.6	08.0	08.7	26.9	18.4	00.3	00.8	13.1	01.0	16.1	00.1
n	29	29	29	29	29	29	29	29	29	29	29
$\bar{X}$	06.8	08.1	09.9	28.2	17.4	00.4	01.3	11.7	00.6	15.4	00.2
SE	00.2	00.2	00.3	00.4	00.3	00.1	00.1	00.3	00.1	00.3	00.0
95% CI	00.4	00.4	00.6	00.7	00.6	00.1	00.2	00.5	00.1	00.6	00.0

Appendix VI continued...

VIAL F

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	07.2	10.8	10.7	24.5	17.4	00.7	01.5	12.4	00.2	14.6	00.0
02	07.3	09.8	09.4	24.6	19.3	01.1	02.6	10.9	00.2	14.7	00.1
03	06.4	09.6	09.4	24.1	16.5	01.3	02.3	12.6	01.4	16.1	00.4
04	05.8	09.6	11.0	26.2	18.2	00.7	02.3	11.0	00.5	14.7	00.0
05	08.9	09.6	12.1	29.6	16.9	00.2	00.8	09.4	00.2	12.2	00.1
06	08.0	08.9	12.2	29.9	14.9	00.4	01.4	09.6	01.1	13.3	00.3
07	08.5	08.7	12.2	29.0	14.6	00.8	02.2	08.3	00.9	14.1	00.7
08	06.2	08.7	07.7	24.3	19.2	00.5	02.4	14.5	00.8	15.4	00.2
09	06.3	08.9	07.9	27.5	16.5	00.5	02.3	13.8	00.9	15.2	00.2
10	06.1	08.4	06.8	24.8	17.9	00.6	02.3	15.2	01.1	16.7	00.2
11	06.2	08.4	07.0	25.2	17.4	00.7	02.3	14.7	01.4	16.4	00.3
12	06.2	07.6	12.6	29.9	17.5	00.0	00.9	11.7	00.4	13.2	00.0
13	07.3	07.5	12.1	32.0	18.0	00.0	00.6	10.3	00.0	12.2	00.0
14	07.1	07.4	10.4	30.1	19.8	00.0	00.6	10.3	00.2	14.1	00.0
15	08.0	08.5	13.6	25.5	18.9	00.0	00.6	10.1	00.1	14.7	00.0
16	05.0	08.4	13.8	29.7	13.7	00.0	01.1	12.7	00.0	17.5	00.0
17	05.3	06.6	14.0	30.4	14.7	00.0	01.2	11.4	00.0	16.4	00.0
18	05.2	06.2	14.1	30.1	13.1	00.2	00.5	12.3	00.0	18.2	00.0
19	05.3	06.7	14.2	32.3	14.3	00.3	00.8	10.8	00.0	15.2	00.0
20	04.7	05.3	10.7	26.7	16.8	00.2	02.5	14.0	00.8	18.2	00.2
21	04.8	05.4	11.6	27.3	16.9	00.2	02.0	14.1	00.7	16.9	00.2
22	04.7	05.3	10.7	26.7	16.8	00.2	02.5	14.0	00.8	18.2	00.2
23	04.8	06.6	11.0	29.5	16.0	00.3	01.3	12.8	00.5	17.1	00.2
24	04.8	06.5	10.2	31.4	15.2	00.3	01.2	12.8	00.4	16.7	00.3
25	06.6	07.3	08.4	33.5	15.4	00.4	01.1	10.1	00.6	16.4	00.2
26	06.7	07.3	08.7	33.9	16.0	00.2	00.7	09.8	00.3	16.3	00.1
27	06.7	07.5	07.9	30.0	18.3	00.4	00.8	11.2	00.3	16.8	00.1
28	06.7	07.8	08.1	30.1	18.1	00.4	00.7	11.0	00.2	16.8	00.1
29	06.3	07.0	07.4	32.4	18.2	00.2	01.2	12.2	00.5	14.4	00.2
30	06.5	07.4	07.6	33.8	18.6	00.2	00.9	10.9	00.3	13.7	00.1
31	06.1	06.7	07.2	34.5	18.3	00.3	01.3	11.3	00.2	13.9	00.2
32	06.1	06.5	07.4	34.1	18.7	00.2	01.1	11.6	00.2	14.0	00.1
33	07.3	08.0	07.9	25.9	20.2	00.3	01.2	11.7	01.3	15.9	00.3
34	07.3	08.0	08.2	26.4	19.7	00.3	01.1	11.4	01.1	16.3	00.2
35	07.3	08.1	08.4	28.6	18.6	00.1	00.7	11.6	00.8	15.7	00.1
n	35	35	35	35	35	35	35	35	35	35	35
$\bar{X}$	06.5	07.8	10.0	29.0	17.2	00.3	01.3	11.7	00.5	15.4	00.2
SE	00.2	00.2	00.4	00.5	00.3	00.1	00.1	00.3	00.1	00.3	00.0
95% CI	00.3	00.4	00.7	00.9	00.5	00.1	00.2	00.5	00.1	00.4	00.0

Appendix VI continued...

VIAL G

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	04.5	08.9	11.5	29.3	19.8	00.0	01.4	10.8	00.0	13.8	00.0
02	06.9	07.7	13.5	27.4	20.5	00.3	01.3	08.5	00.0	13.9	00.0
03	07.0	07.4	14.6	27.8	19.0	01.2	01.1	08.8	00.0	13.1	00.0
04	07.3	08.2	12.0	28.3	19.9	00.0	00.8	09.0	00.0	14.5	00.0
05	06.6	08.4	09.7	26.6	17.8	00.2	02.2	12.6	00.1	15.9	00.0
06	06.5	08.2	09.2	28.8	17.7	00.4	02.3	11.6	00.4	15.2	00.0
07	07.2	09.1	08.6	26.5	22.5	00.0	00.9	10.5	00.0	14.7	00.0
08	06.4	10.0	09.3	29.2	21.7	00.2	00.9	10.0	00.0	12.2	00.0
09	06.2	06.5	11.7	33.8	16.1	00.0	01.6	10.7	00.3	13.1	00.0
10	06.7	06.8	13.2	34.6	14.0	00.0	01.4	10.1	00.2	13.0	00.0
11	05.8	06.2	11.0	29.2	11.0	00.0	01.1	11.0	00.2	14.5	00.0
12	06.3	06.7	13.4	33.3	15.1	00.0	00.9	10.1	00.1	14.1	00.0
13	05.2	05.3	13.5	29.7	13.7	00.0	00.6	14.8	00.3	16.9	00.0
14	05.6	05.6	13.2	32.3	14.7	00.0	00.7	12.3	00.2	15.4	00.0
15	05.3	06.1	14.6	30.0	14.0	00.0	00.5	12.7	00.2	16.6	00.0
16	05.5	06.8	13.8	34.2	14.1	00.0	00.7	10.4	00.0	14.6	00.0
17	04.5	06.0	10.0	31.0	15.1	00.2	01.6	13.1	00.5	17.6	00.3
18	04.6	05.4	10.9	27.4	17.8	00.1	01.4	13.4	00.3	18.5	00.2
19	05.1	05.9	11.8	29.3	15.4	00.2	01.8	12.7	00.4	17.4	00.1
20	04.5	05.0	11.6	27.7	17.1	00.1	01.3	13.6	00.2	18.8	00.1
21	07.8	08.4	09.2	29.1	15.9	00.3	01.4	11.3	00.4	15.3	00.1
22	07.8	08.2	09.3	29.5	15.7	00.3	01.3	11.3	00.9	15.5	00.2
23	07.4	08.0	09.6	28.1	16.2	00.3	01.1	12.4	00.5	16.3	00.1
24	07.3	08.2	09.5	27.0	16.3	00.3	01.0	13.2	00.6	16.4	00.2
25	07.7	08.3	08.6	31.4	17.6	00.0	01.1	10.5	00.0	14.8	00.0
26	07.6	08.4	08.6	30.6	18.0	00.0	00.9	10.7	00.0	15.2	00.0
27	07.9	08.4	08.7	27.2	18.4	00.2	01.1	11.3	01.0	15.7	00.1
28	07.7	08.2	08.6	27.5	18.5	00.2	01.2	11.2	00.9	15.8	00.2
n	28	28	28	28	28	28	28	28	28	28	28
$\bar{X}$	06.4	07.4	11.0	29.5	16.9	00.2	01.2	11.4	00.3	15.3	00.1
SE	00.2	00.3	00.4	00.4	00.5	00.0	00.1	00.3	00.1	00.3	00.0
95% CI	00.4	00.4	00.7	00.8	00.8	00.1	00.1	00.5	00.1	00.5	00.0

Appendix VI continued...

VIAL H

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	07.2	09.7	09.3	29.6	19.4	00.0	00.7	10.9	00.0	13.2	00.0
02	07.6	10.0	09.4	28.3	20.9	00.0	00.7	11.0	00.0	12.1	00.0
03	06.7	11.7	10.3	29.6	20.5	00.0	01.4	08.8	00.0	11.1	00.0
04	06.3	09.7	08.9	32.5	18.4	00.0	00.9	10.1	00.0	13.2	00.0
05	08.4	09.1	14.2	23.4	19.4	00.0	01.0	09.4	00.0	15.1	00.0
06	07.6	08.5	13.7	26.2	20.1	00.0	00.6	09.4	00.0	13.9	00.0
07	07.4	08.3	08.0	29.2	17.6	00.0	00.5	13.8	00.0	15.2	00.0
08	07.2	08.1	08.5	29.1	17.9	0.00	00.6	13.4	00.0	15.2	00.0
09	06.7	08.1	09.0	28.3	19.7	00.0	01.1	12.4	00.0	14.7	00.0
10	07.0	08.4	08.6	31.8	18.9	00.0	00.8	10.6	00.0	13.9	00.0
11	05.4	06.2	13.0	35.6	20.6	00.0	01.0	08.6	00.0	10.6	00.0
12	05.6	06.9	12.9	33.6	21.0	00.0	00.7	08.3	00.0	11.0	00.0
13	06.0	07.4	11.6	35.7	16.4	00.0	00.8	09.2	00.3	12.6	00.0
14	06.4	07.5	13.7	32.6	19.1	00.0	00.6	08.1	00.1	11.9	00.0
15	05.5	06.9	12.9	28.4	16.4	00.0	01.4	12.3	00.3	15.7	00.0
16	05.6	07.3	12.2	30.1	16.2	00.0	01.0	11.9	00.2	15.5	00.0
17	05.0	06.2	12.8	30.0	15.3	00.0	01.3	13.0	00.1	16.3	00.0
18	05.0	05.7	12.7	29.4	16.8	00.0	00.6	13.4	00.0	16.4	00.0
19	05.0	06.4	11.5	29.0	16.8	00.4	01.1	11.0	00.1	18.6	00.2
20	04.9	05.7	10.7	30.2	17.6	00.0	00.7	11.2	00.0	18.9	00.1
21	04.7	05.6	11.1	30.3	18.4	00.1	00.4	11.5	00.0	18.1	00.0
22	04.1	05.0	09.9	35.0	15.7	00.0	00.1	11.6	00.0	18.6	00.0
23	06.7	07.5	07.7	29.5	15.9	00.0	01.7	12.0	00.7	18.3	00.0
24	06.8	07.3	07.7	29.5	16.3	00.0	01.6	12.1	00.4	18.3	00.0
25	06.7	07.2	07.9	28.5	17.2	00.0	01.1	12.6	00.6	17.9	00.0
26	06.6	07.0	08.1	28.6	17.2	00.0	01.3	12.6	00.6	18.0	00.0
27	07.4	07.9	08.5	28.0	17.3	00.0	00.7	12.9	00.0	17.3	00.0
28	07.4	08.0	08.3	27.0	17.3	00.0	00.9	12.6	00.7	17.8	00.0
n	28	28	28	28	28	28	28	28	28	28	28
$\bar{X}$	06.4	07.6	10.3	29.8	17.9	00.0	00.9	11.3	00.2	15.5	00.0
SE	00.2	00.3	00.4	00.5	00.3	00.0	00.1	00.3	00.0	00.5	00.0
95% CI	00.3	00.5	00.7	00.8	00.5	00.0	00.1	00.5	00.1	00.8	00.0

Appendix VI continued...

VIAL I

No.	C7	C6	C5	C4	C3	C2	C1	A1	A2	A3	A4
01	08.3	09.4	13.4	29.3	18.1	00.0	00.7	08.2	00.0	12.6	00.0
02	07.9	09.0	12.8	29.9	17.6	00.0	01.3	08.4	00.0	13.1	00.0
03	06.6	09.2	14.0	33.4	17.6	00.0	00.5	08.2	00.0	10.5	00.0
04	05.7	09.8	14.1	31.4	17.7	00.0	00.7	09.4	00.0	11.3	00.0
05	07.3	08.1	08.9	30.0	19.4	00.0	00.7	12.2	00.0	13.4	00.0
06	06.8	07.4	08.3	31.9	18.7	00.0	01.0	11.8	00.0	14.1	00.0
07	06.7	07.6	08.0	30.5	19.6	00.0	01.3	11.8	00.0	14.5	00.0
08	06.8	07.4	07.7	32.9	18.4	00.0	01.2	12.0	00.0	13.6	00.0
09	06.8	07.5	09.3	28.3	19.3	00.0	01.1	10.4	00.0	17.3	00.0
10	06.7	07.4	08.8	26.7	19.4	00.0	01.6	11.2	00.0	18.2	00.0
11	06.7	07.5	09.0	25.9	19.5	00.0	01.6	11.5	00.0	18.3	00.0
12	07.4	07.6	09.2	27.1	17.2	00.0	01.3	12.3	00.3	17.4	00.0
13	07.0	07.8	09.4	27.5	19.1	00.0	01.3	10.6	00.0	17.3	00.0
14	07.6	08.1	09.3	27.6	16.9	00.0	00.9	12.0	00.0	17.6	00.0
15	07.6	08.1	09.3	28.1	18.3	00.0	00.8	11.1	00.0	16.7	00.0
16	07.7	07.6	09.5	28.5	18.0	00.0	00.8	11.5	00.0	16.4	00.0
n	16	16	16	16	16	16	16	16	16	16	16
$\bar{X}$	07.1	08.1	10.1	29.3	18.4	00.0	01.1	10.8	00.0	15.1	00.0
SE	00.2	00.2	00.5	00.6	00.2	00.0	00.1	00.4	00.0	00.6	00.0
95% CI	00.3	00.4	01.0	01.0	00.4	00.0	00.1	00.6	00.0	01.1	00.0